MICROBIAL LIPID ACCUMULATION THROUGH BIOREMEDIATION OF PALM OIL MILL EFFLUENT BY CO-CULTURING YEAST AND BACTERIA

MD AHASANUL KARIM

DOCTOR OF PHILOSOPHY

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

DECLARATION OF THESIS AND COPYRIGHT

Author's Full Name : MD AHASANUL KARIM

Date of Birth : 15.12.1988

Title : MICROBIAL LIPID ACCUMULATION THROUGH BIOREMEDIATION OF PALM OIL MILL EFFLUENT BY CO-CULTURING YEAST AND BACTERIA

Academic Session : SEMESTER I 2020/2021

I declare that this thesis is classified as:

☐	CONFIDENTIAL	(Contains confidential information under the Official Secret Act 1997)*
☐	RESTRICTED	(Contains restricted information as specified by the organization where research was done)*
☒	OPEN ACCESS	I agree that my thesis to be published as online open access (Full Text)

I acknowledge that Universiti Malaysia Pahang reserves the following rights:

1. The Thesis is the Property of Universiti Malaysia Pahang
2. The Library of Universiti Malaysia Pahang has the right to make copies of the thesis for the purpose of research only.
3. The Library has the right to make copies of the thesis for academic exchange.

Certified by:

(Student's Signature) (Supervisor's Signature)

BW0594411 Dr. Che Ku Mohammad Faizal

New IC/Passport Number Name of Supervisor

Date: Date:

NOTE : * If the thesis is CONFIDENTIAL or RESTRICTED, please attach a thesis declaration letter.

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis, and, in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Doctor of Philosophy.

_____

(Supervisor's Signature)

Full Name : DR. CHE KU MOHAMMAD FAIZAL

Position : ASSOCIATE PROFESSOR

Date : DECEMBER 16, 2020

_____

(Co-supervisor's Signature)

Full Name : DR. MD. MAKSUDUR RAHMAN KHAN

Position : PROFESSOR

Date : DECEMBER 16, 2020

_____

(Co-supervisor's Signature)

Full Name : DR. ABU YOUSUF

Position : PROFESSOR

Date : DECEMBER 16, 2020

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Malaysia Pahang or any other institutions.

(Student's Signature)

Full Name : MD AHASANUL KARIM

ID Number : PKE16005

Date : DECEMBER 16, 2020

MICROBIAL LIPID ACCUMULATION THROUGH BIOREMEDIATION OF PALM OIL MILL EFFLUENT BY CO-CULTURING YEAST AND BACTERIA

MD AHASANUL KARIM

Thesis submitted in fulfillment of the requirements
for the award of the degree of
Doctor of Philosophy

Faculty of Chemical and Process Engineering Technology
UNIVERSITI MALAYSIA PAHANG

DECEMBER 2020

ACKNOWLEDGEMENTS

The research presented in this thesis has been carried out at the Faculty of Engineering Technology, Universiti Malaysia Pahang, Malaysia. The journey of my research would not be possible without help and support of several persons.

At first, I wish to my humble gratefulness and sincere thanks to honorable supervisor, Associate Professor Dr. Che Ku Mohammad Faizal for his valuable support and guidance throughout the study. Indeed, it was utmost opportunity for me to get him as my main supervisor. He has always been able to keep on amazing me with his introspective thinking, ideas and insights. I would also like to give special thanks to my other supervisors, Professor Dr. Md. Maksudur Rahman Khan and Professor Dr. Abu Yousuf for their ideas, valuable inputs, inspiration and support.

I am highly grateful to the Universiti Malaysia Pahang (UMP), Malaysia, for funding this work under the grant number of FRGS RDU 160150 and PRGS 180317. Apart from that, I would like to acknowledge the authority LKPP Corporation Sdn. Bhd., Gambang, Pahang, Malaysia for their continuous support by giving me palm oil mill effluent and anaerobic sludge samples.

I would like to express my sincere gratitude to Dr. M. Amirul Islam for his unconditional support for his kind helps and suggestions to conduct my experiments and to write-up my thesis as well as research paper. Special thanks also go to Dr. Puranjan Mishra for helping me to do the optimization by Response Surface Methodology. I also greatly appreciate to Miss. Fatin Zafirah Mansur and Miss. Nur Ain Zakiah Mohd Yusof for correcting and polishing my thesis abstract (Malay version). Special thanks must be given to Mr. Zaied bin Khalid for giving me support for thesis formatting and in dealing with official matters.

Lastly, I also want to thank to my parents and family for their continuous supports, encouragements and inspirations. It would be tough to finish my study without their supports and devotions.

ABSTRAK

Pelepasan efluen kilang kelapa sawit (POME) ke tanah pertanian menyebabkan pencemaran alam sekitar kesan daripada ketinggian kepekatan komponen fenolik, keperluan oksigen kimia (COD) dan keperluan oksigen biokimia (BOD). Malah, penyusutan progresif bahan api fosil dan sumber mineral turut dikenal pasti sebagai cabaran pada masa hadapan. Kaedah penghasilan lipid mikrobial secara serentak melalui rawatan air sisa berpotensi menjadi penyelesaian terhadap kedua-dua isu tersebut. Kajian ini bertujuan untuk menghasilkan lipid microbial menggunakan bakteria dan yis oleaginous yang kuat iaitu *Bacillus cereus* (*B. cereus*) dan *Lipomyces starkeyi* (*L. starkeyi*) melalui bioremediasi POME dengan penapaian secara berkelompok. Kepekatan substrat POME yang berbeza (25%, 50%, 75%, dan 100%) digunakan sebagai nutrien untuk mengkaji tahap optimum kepekatan POME bagi pengeluaran hasil biojisim dan lipid paling maksimum. Hasil pemerhatian menunjukkan larutan POME yang mempunyai kepekatan sederhana (POME 50%) mempunyai kadar pertumbuhan mikrobial dan pengumpulan lipid yang lebih tinggi serta tahap bioremediasi yang signifikan. Tahap bioremediasi dinilai menggunakan beberapa parameter sisa air (BOD, COD, jumlah fenol, jumlah karbon organik dan lain-lain) dan indeks percambahan benih (GI) kacang hijau (*Vigna radiata*). POME yang dirawat menggunakan gabungan inokulum kultur (*B. cereus* dan *L. starkeyi*) menunjukkan penurunan ketara kadar pencemaran, khususnya COD bagi POME 50%, iaitu kecekapan penyingkiran sebanyak 83.66%. POME tersebut turut mencapai nilai GI yang lebih tinggi berbanding sampel yang lain (dirawat menggunakan kultur tulen dan tanpa rawatan) kesan daripada remediasi yang ketara terhadap kehadiran organik berbahaya di dalam POME seperti yang dibuktikan oleh analisis Gas Kromatografi-Spektometri Jisim (GC-MS). Gabungan inokulum kultur telah menyumbang kepada pertumbuhan biojisim tertinggi (9.16 g/L) dan penghasilan lipid (2.21 g/L), dengan kandungan lemak 24.12% (berasaskan berat kering) dalam 50% (v/v) POME. Komposisi lipid dianalisa berdasarkan metil ester asid lemak menggunakan GC-MS. Kajian mendapati bahawa C16 dan C18 adalah asid lemak utama dalam lipid inokulum kultur yang membolehkan lipid mikrobial dapat digunakan sebagai bahan biodiesel. Satu kaedah baru pengekstrakan lipid, iaitu elektroporasi (EP) telah digunakan untuk mengekstrak lipid mikrobial dan kecekapan EP turut dibandingkan dengan beberapa kaedah konvensional yang lain. EP menunjukkan tahap kecekapan pengekstrakan lipid yang lebih tinggi sebanyak 31.88% (wt.%) berbanding dengan kaedah ultrabunyi (11.89%), reagen Fenton (16.80%), dan pengekstrakan pelarut (9.60%). Pengaruh parameter kajian seperti komposisi inokulum, pH, suhu, dan masa bagi penilaian kecekapan proses penyingkiran COD dan penghasilan lipid dioptimumkan menggunakan kaedah rangsangan permukaan. Pengoptimuman gabungan inokulum kultur menunjukkan bahawa komposisi inokulum, pH, suhu, dan masa mempunyai kesan yang signifikan terhadap prestasi penyingkiran COD dan pengumpulan lipid. Kecekapan maksimum penyingkiran COD sebanyak 86.54% dan pengumpulan lemak 2.95 g/L tercapai dengan komposisi inokulum, pH, suhu, dan masa inkubasi masing-masing adalah 50:50, 6.50, 32.5 ℃ dan 90 h. Oleh itu, hasil kajian ini menunjukkan bahawa gabungan kultur *B. cereus* dan *L. starkeyi* adalah inokulum yang berpotensi untuk mencapai pertumbuhan biojisim dan penghasilan lipid yang lebih tinggi dengan bioremediasi POME. Pendekatan kaedah gabungan bagi mencapai dwi-objektif kajian (bioremediasi POME dan penghasilan lipid mikrobial) memberikan sebuah strategi baru kepada pengilang minyak kelapa sawit.

ABSTRACT

The discharge of palm oil mill effluent (POME) on arable land causes large amounts of environmental distress due to its high concentration of phenolic compounds, chemical oxygen demand (COD), and biochemical oxygen demand (BOD). On the other hand, the progressive depletion of fossil fuels and mineral resources have also been identified as a future challenge. The approach of simultaneous microbial lipid production through the wastewater treatment could be a potential option to address both renewable energy production and environmental resilience. This study aims to produce microbial lipids using robust oleaginous bacteria and yeast of *Bacillus cereus* (*B. cereus*) and *Lipomyces starkeyi* (*L. starkeyi*) through the bioremediation of POME in batch mode fermentation. Different concentrations of POME substrates (25%, 50%, 75%, and 100%) were used as nutrients to determine the optimum POME concentration for achieving maximum yield of biomass as well as lipid production. It was observed that among the different dilutions, the moderately diluted solution of POME (50% POME) showed higher microbial growth and lipid accumulation and offered a significantly higher degree of bioremediation. The degree of bioremediation was assessed by evaluating several wastewater parameters (*i.e.*, BOD, COD, total phenol, total organic carbon, *etc.*) and determining the seed germination index (GI) of Mung bean (*Vigna radiata*). POME treated with a co-culture inoculum (*B. cereus* and *L. starkeyi*) substantially reduced the pollution load, particularly, in COD for 50% POME, thus demonstrating a removal efficiency of 83.66%. Furthermore, POME treated with co-culture inoculum obtained a higher GI value than the other samples (treated by pure cultures and untreated) due to the significant remediation of detrimental organics present in the POME as evidenced by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Nevertheless, the co-culture inoculum was found to have potential for the highest biomass growth (9.16 g/L) and lipid accumulation (2.21 g/L), with a lipid content of 24.12% (dry weight basis) in the 50% (v/v) POME. Lipid composition was analyzed in terms of fatty acid methyl esters using GC-MS. C16 and C18 were found to be the predominant fatty acids in the lipid of co-culture inoculum suggesting the potential of microbial lipid to be used as a biodiesel feedstock. A novel lipid extraction method, namely electroporation (EP) was used to extract microbial lipid and the efficiency of EP was compared with some other conventional methods. The EP demonstrated a higher lipid extraction efficiency of 31.88% (wt.%) compared to the ultrasound (11.89%), Fenton's reagent (16.80%), and solvent extraction (9.60%). Finally, the influence of several process parameters such as inoculum compositions, pH, temperature, and time on the performance of the COD removal efficiency and lipid accumulation were optimized using response surface methodology. Optimization of co-culture inoculum showed that the inoculum composition, pH, temperature, and time had a significant effect on the performance of the COD removal and lipid accumulation. The maximum COD removal efficiency of 86.54% and lipid accumulation of 2.95 g/L could be obtained while the inoculum composition, pH, temperature, and incubation time were 50:50, 6.50, 32.5 ℃, and 90 h, respectively. Therefore, the results of this study suggest that the co-culture of *B. cereus* and *L. starkeyi* could be a promising inoculum for attaining higher biomass growth and lipid production in conjunction with the bioremediation of POME. This combined approach of achieving dual objectives (bioremediation of POME and microbial lipid production) that is utilized in the present study provides a novel strategy for palm oil millers.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

V	Voltage
Hz	Hertz
SD	Standard Deviation
°C	Degree Celsius
Min	Minute
cm	Centimeter
D	Diameter
d	Days
g	Gram
s	Second
h	Hour
kg	Kilogram
L	Length
M	Micro Mole
m^3	Cubic Meter
V	Volume
µ	Micro (10^{-6})
C	Carbon
N	Normality
kV	Kilovolts (10^3)
µs	Microsecond (10^{-6})
ms	Millisecond (10^{-3})
J	Joule
L	Liter
mL	Milliliter
π	Pi
σ	Sigma
atm	Standard atmosphere
Ω	Ohm
kJ	Kilojoule
W	Watt

kW	Kilowatt (10^3)
f	Frequency
T	Period
Σ	Summation
b_i	Linear coefficient
b_o	Constant coefficient
b_{ii}	Quadric coefficient
b_{ij}	Interaction of coefficient, x_i, x_j coded values
x_1	Concentration of inoculum A
x_2	Substrate pH
x_3	Temperature
x_4	Operational time
y_1	COD removal efficiency
y_2	Lipid accumulation

LIST OF ABBREVIATIONS

GHGs	Greenhouse Gases
AD	Anaerobic Digestion
VS	Volatile Solid
AS	Activated Sludge
BOD	Biochemical Oxygen Demand
TAGs	Triacylglycerols
COD	Chemical Oxygen Demand
PUFA	Polyunsaturated Fatty Acids
C/N	Carbon to Nitrogen Ratio
HRT	Hydraulic Retention Time
H_2O_2	Hydrogen Peroxide
OLR	Organic Loading Rate
SCOs	Single-cell Oils
POME	Palm Oil Mill Effluent
TI	Treatment Intensity
DAGs	Diacylglycerols
SS	Suspended Solids
TN	Total Nitrogen
TOC	Total Organic Carbon
TPC	Total Phenolic Content
TSS	Total Suspended Solids
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids
NN	Nitrate Nitrogen
AN	Ammoniacal Nitrogen
TS	Total Solids
TDS	Total Dissolved Solids
CH_4	Methane
CO_2	Carbon Dioxide
CO	Carbon Monoxide
DC	Direct Current

OD	Optical Density
EP	Electroporation
IRE	Irreversible Electroporation
RE	Reversible Electroporation
GC-MS	Gas Chromatography and Mass Spectrophotometry
FESEM	Field Emission Electron Microscopy
FAME	Fatty Acid Methyl Esters
CCD	Central Composite Design
SMR	Steam Methane Reforming
WGSR	Water Gas Shift Reaction
FFAs	Free Fatty Acids
FAME	Fatty Acid Methyl Ester
FAAE	Fatty Acid Alkyl Ester
DNA	Deoxyribonucleic Acid
PEF	Pulsed Electric Field
SCOD	Soluble Chemical Oxygen Demand
TCOD	Total Chemical Oxygen Demand
ECP	Exocellular Polymers
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
FD	Factorial Design
ANOVA	Analysis of Variance
OFAT	One Factor at A Time
BBD	Box-Behnken Design
PBD	Placket-Burman Design
DoE	Design of experiment
RSM	Response Surface Methodology
CFU	Colony Forming Units
DO	Dissolved Oxygen
GI	Germination Index
PMW	Pulse-Width Modulation
IDE	Arduino Software
DI	Deionization

LB	Luria Bertani
mV	Millivolt
rpm	Revolutions per Minute
mM	Milli Mole
mg/L	Milligram per liter
g/L	Gram per liter
APHA	American Public Health Association

CHAPTER 1

INTRODUCTION

1.1 Background of the Research

The modern world is confronting several burning issues such as energy crisis, wastewater generation, air pollution and global warming. However, environmental pollution caused by excessive wastewater generation and depletion of energy are the most important for human society (Chowdhary et al., 2018). Moreover, the increase in worldwide energy consumption due to population growth and economic development is becoming greater day by day (Schneider et al., 2013). To meet our energy needs, industrialization is necessary and hence, an overwhelming amount of industrial wastewater has been generated (Baranitharan et al., 2015). Mostly non-renewable fossil fuels such as petroleum, coal and natural gas are being used to fulfill this demand (Nayak et al., 2016). Generally, fossil fuels include about 88% of the global energy consumption which constitute oil (35%), coal (29%) and natural gas (24%) as the major fuels. Nuclear energy and hydroelectricity cover another 5–6% of the global energy consumption (Brennan & Owende, 2010). The use of fossil fuels as energy sources is unsustainable due to limited resources and accumulation of greenhouse gases (GHGs) in the environment (Ahmad et al., 2016). Compounding the problem further, the reserve of fossil fuel and mineral resources is depleting more than it can meet the demand from the population growth and industrialization (Nayak et al., 2016). In 2030, the world will be in need of almost 60% more energy than what is needed today (Ahmad et al., 2016). Therefore, the global research community is looking for substitutes for nonrenewable energy sources to meet energy demands and support an eco-friendly environment. In the past decade, intensive research efforts have been dedicated to finding alternative renewable energy sources, particularly energy generation, through a sustainable treatment of wastewater (Li et al., 2014; Nayak et al., 2016). A number of renewable energy sources, such as biodiesel, biogas, bioethanol, biohydrogen, etc., have been identified as potential options to replace the typical fossil fuels (Yousuf et al., 2017d). According to the European Renewable Energy Council, around 50% of the global energy

supply will be supported by renewable energy by 2040 (Sun et al., 2016). In this context, the ideal solution would be to use wastewater as a source of renewable energy, which would address both wastewater treatment and energy depletion issues.

The wastewater, palm oil mill effluent (POME) is one of the major pollutants in Southeast Asia. Specifically, the production of palm oil is abundant in Indonesia, Malaysia, and Thailand, which produce a large volume of effluents with a high organic carbon content (Bala et al., 2015). Several reports have shown that these values are 100 times higher than those of municipal sewage (Iwuagwu & Ugwuanyi, 2014). In Malaysia, the palm oil industry has grown by leaps and bounds over the last five decades and the number of palm oil mills was 454 with an estimated palm oil production of 19.9 million tonnes in 2017 (Chan & Chong, 2019; Chin et al., 2013). POME is a low pH wastewater, containing a high level of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) in the range of 25,000–54,000 mg/L and 50,000 to >100,000 mg/L, respectively. POME is often discharged directly to the environment from a mill, which is objectionable and could pollute streams, rivers, and the surrounding lands (Choong et al., 2018). When POME is discharged into water bodies, it turns the water brown, smelly, and slimy, and causes de-oxygenation that may kill fish and other aquatic organisms (Islam et al., 2017b). The disposal of untreated POME into soil alters its physicochemical properties and nutritional status and causes undesirable decreases in pH and increases in salinity (Islam et al., 2017a). In addition, environmental pollutants such as heavy metals, high amounts of ammonia, phenolic compounds, large concentrations of organic contents and low pH severely affect plant seed germination as well as root elongation (Gopalakrishnan et al., 2015). The soil quality can be deteriorated, and seed germination can be slowed down due to raw POME discharge. With that goal in mind, POME must be treated before it is discharged into the natural environment. Therefore, different techniques have been used for POME treatment before disposal. Generally, ponding-based anaerobic and aerobic digestion are the most commonly used techniques for POME treatment. The ponding system has low maintenance costs due to operational and process simplicity; therefore, it is considered to be a feasible means of treating high strength organic wastewater (Ismail et al., 2013; Lek et al., 2018). However, it entails a long hydraulic retention time (~ 40 to 200 days) and a large land area (1 to 5 ha). In general, the existing POME treatment strategy requires several stages, hence, it is an intensive and expensive process with high energy demand and in many cases, not the most sustainable

option for economic and environmental reasons (Nizami et al., 2017). Currently, significant amounts of wastewater are being treated using different techniques (i.e., coagulation and flocculation, sedimentation, adsorption, filtration, photocatalytic treatment, aerobic and anaerobic digestion, etc.), which are considered either energy intensive or environmentally unfriendly (Alhaji et al., 2016). In this quest, an eco-friendly treatment method is required to develop a no-loss waste treatment process (Iwuagwu & Ugwuanyi, 2014).

The biofuels (e.g., biochar, biogas, biohydrogen, biodiesel, bioethanol, etc.) produced from renewable sources (e.g., wood and wood residues, plants, animal matters, wastewater feedstocks, algae or algae derived biomass, etc.) have been considered as sustainable renewable sources to meet the future energy demand (Bharte & Desai, 2018). Among the different biofuels, the biodiesel is regarded as a promising alternative to the petroleum-based fuels, especially, for the transportation sector. Conventional biodiesel is produced by the transesterification of triacylglycerols (TAGs) from vegetable oils and animal fats with short chain alcohols, usually, methanol or ethanol (Kirubakaran & Selvan, 2018). Currently, the biodiesel is produced from edible substances (such as corn, soybean, rapeseed, and sugarcane) and plant feedstocks (like Jatropha, Miscanthus and switchgrass). However, these sources of triglycerides cannot satisfy the demand for biodiesel production due to the food versus feed competition and the limited availability of cultivable land for edible and non-edible feedstocks. Moreover, a significant amount of crude glycerol is produced as a by-product of this process and creates a bioburden to the environment (Kirubakaran & Selvan, 2018). In addition, the cost of the biodiesel, mainly due to the vegetable oils used as feedstocks, still exceeds that of the mineral diesel properties (Pinzi et al., 2014). Consequently, alternative sources of TAGs are required. In recent years, microbial lipids, also known as single-cell oils (SCOs), have piqued industrial interest due to their particular and precise biochemical and physicochemical properties (Kirubakaran & Selvan, 2018). The microbial lipids are regarded as promising alternatives to vegetable oils used for biodiesel production since the oil properties are similar in type, composition, and structure to the fatty acids (Kumar et al., 2020). Certain microorganisms (like algae, yeast, mold, bacteria, and fungi) are capable of accumulating more than 20% of lipids inside their cells as TAGs, which are known as oleaginous microorganisms (Kumar et al., 2020). Generally, the lipid yield of common oil crops is very poor. For example, canola yields only 1200 L/ha and palm oil approximately 6000

L/ha because the plant oil content is often less than 5% of their biomass. In contrast, some microorganisms achieve a much higher oil content but do not require fertile land. Furthermore, they are not affected by the seasons and the climate (Chen et al., 2018). In addition, microbial oils could be produced in a much shorter time than vegetable oils due to the short life cycle of microbes and the comparatively smaller amount of space and labor needed. However, in the case of microbial lipid production, the key setback is the cost of the growth medium (raw material cost) for cultivating microbes, which is up to 80% of the total production cost (Patel et al., 2020). Therefore, exploring other carbon sources instead of glucose is imperative to reduce the cost of microbial oils, especially, for such oils used in biodiesel production. The use of industrial wastewater like POME as a feedstock to produce microbial lipids could be an ideal solution, as it simultaneously addresses the need for renewable carbon fuels and the reduction of the environmental burden posed by palm oil milling.

1.2 Problem Statement

Current scenario of POME treatment pointed out a necessity to investigate other non-traditional ways of POME valorization. Oleaginous microbes including bacteria (Patel et al., 2020), yeasts (Pirozzi et al., 2015), molds (Patel et al., 2020), and algae (Dong et al., 2016) are capable of converting several wastewater like POME into lipids (Pirozzi et al., 2014). Several studies have been reported to treat POME using various microbes, however, most of the techniques focused on bioremediation of POME only but not simultaneous valorization of POME (Bala et al., 2015; Ganapathy et al., 2019). In addition, the yield of lipid production is comparatively lower while pure cultures of microbes were used (Kumar et al., 2020; Patel et al., 2020). Usually, environmental stress conditions, like nutrition deficiency, are produced for oleaginous microbes to obtain high lipid content. However, the high lipid content achieved through this way using monocultures is often offset by decreased growth rates, that leads to lower biomass and lipid productivity (Magdouli et al., 2016). Lipid production performance could significantly be enhanced using different technique particularly by metabolically engineered strain production (Xu et al., 2016; Yang et al., 2018); however, the proliferation and stability of modified microbes may be sensitive in complex medium, especially, while an industrial wastewater is used as a nutrient source. Moreover, monocultures are associated with high risk of contamination which results in product and

capital losses during production process. Several symbiotic co-cultures possess traits that could overcome not only these limitations but also boost up in biomass, lipid, and other products yield, though not universally applicable to all cell systems (Padmaperuma et al., 2018). In this context, a sustainable approach is highlighted in the present study with a possibility of valorizing POME to microbial lipid and concurrent bioremediation of POME using oleaginous microorganisms that could add a net positive value to this waste material from the environmental point of view.

The cell wall disruption is the most challenging part of lipid extraction process from microbial cells, since lipids are located inside the cell (Liu et al., 2016). Numerous disruption methods including physical, chemical and enzymatic have been developed to achieve selective release of biomolecules (Middelberg, 1995; Yusaf & Al-Juboori, 2014). The non-mechanical methods are often limited to small scale and usually result in less intracellular product release and low process efficiency (Günerken et al., 2015; Liu et al., 2016). The chemical-based method e.g., Bligh-Dyer is mostly used for lipid extraction because of its simple and easy operating procedure; however, practical application could be restricted due to various difficulties, such as extensive chemical usage, lower product yield, higher retention time, etc. Additionally, the practice of using chemicals and enzymes can lead to greater complexity in minimizing environmental impact (Liu et al., 2016). Consequently, those have found limited commercial application to date (Günerken et al., 2015). Though mechanical methods such as high-pressure homogenization (Clarke et al., 2010), ultrasonication (Chemat & Khan, 2011), osmotic shocks (Yousuf, 2012) are popular, they are associated with high energy input (Günerken et al., 2015), high heat generation (Byreddy et al., 2015) and time consumption (Liu et al., 2016). Moreover, some of these methods can destroy the biomolecules of interest (denaturation of proteins) (Byreddy et al., 2015; Liu et al., 2016). Therefore, a new cell disruption approach is required to enhance lipid extraction from microbial biomass within a short time and in an eco-friendly manner. Recently, a cell disruption approach called electroporation (EP) is proposed to extract lipid from microbial biomass for the direct transesterification process (Yousuf et al., 2017b). However, the application of irreversible EP to damage cell wall to enhance lipid extraction from the yeast and bacteria have not been studied in detail.

In co-culture system, the composition of co-culture inoculum, could have significant influence on the growth performance and lipid accumulation. Besides the

inoculum compositions, experimental conditions such as pH, time and temperature strongly influence the performance of microbial lipid production. In some recent studies, it has been observed that lipid content and fatty acid compositions vary depending on the nature of microorganisms and the environmental conditions including substrate type and concentration, incubation temperature and period, medium pH, static and shaking condition, nutrients etc. (Subhash & Mohan, 2014; Wang et al., 2015b). Therefore, optimization of operational parameters is emergent to enhance the performance of microbial lipid production by increasing the process efficiency in the optimized conditions. However, optimization of inoculum compositions along with the afore-mentioned parameters (i.e., pH, temperature, time) which determine the performance of lipid accumulation in a yeast-bacteria co-culture has not been studied.

1.3 Research Objectives

The main objective of this study is to focus on the valorization of POME for producing microbial lipid through the bioremediation by using yeast-bacteria co-culture and to investigate the efficiency of microbial lipid extraction using EP technique. The specific objectives of this study are the following:

1. To investigate the bioremediation efficiency of POME by *B. cereus*, *L. starkeyi* and their co-culture inoculum.
2. To study the efficiency of microbial lipid extraction using a novel EP technique compared to several conventional extraction techniques.
3. To evaluate lipid accumulation performance of *B. cereus*, *L. starkeyi* and their co-culture through POME bioremediation.
4. To optimize the yeast-bacteria co-culture for enhancing lipid production and bioremediation efficiency (COD removal) using response surface methodology (RSM).

1.4 Scope of the Study

The scope of this study is discussed as following based on each objective:

The raw POME samples were collected and characterized with different concentrations (25, 50, 75, and 100% POME) to evaluate the optimum POME

concentration in terms of microbial growth profile. The performance of *B. cereus*, *L. starkeyi*, and their co-culture in remediating the pollutants from POME were investigated to elucidate the efficiency of co-culture consortia of yeast-bacteria compared to monocultures. Several wastewater parameters such as BOD, COD, total phenolic content (TPC), total organic carbon (TOC), total nitrogen (TN), nitrate nitrogen (NN), ammoniacal nitrogen (AN), total solids (TS), total suspended solids (TSS), total dissolved solids (TDS), and oil and grease were determined to calculate the removal efficiencies by different inoculums in the targeted POME concentration. The seed germination profiles were studied for untreated (raw) and treated (after digestion by different inoculums) POME to investigate the bioremediation efficiency and their influence on the plant environment. Furthermore, the treated and untreated POME samples were characterized using gas chromatography and mass spectrophotometry (GC-MS), to evaluate pollutant remediation from POME by *B. cereus*, *L. starkeyi* and their co-culture inoculum.

To evaluate the efficiency of EP technique, lipid extraction was carried out using several conventional methods such as solvent extraction, Fenton's method, ultrasound. An EP circuit (comprised high voltage power supply, pulse generator and switching circuit) and an EP reactor were designed and fabricated for EP treatment. The optimal conditions of EP such as voltage (4 kV), frequency (0-100 Hz), treatment time (0 to 10 min), and distance between electrodes (2, 4, and 6 cm) in the reactor were investigated for EP technique by evaluating the microbial cell disruption and the lipid extraction yield. Finally, the lipid extraction yield of EP technique was compared with other methods to justify the efficiency of EP.

The biomass harvesting, lipid accumulation capacity, and productivity in different POME concentrations (25, 50, 75, and 100%) were evaluated to determine the performance of *B. cereus*, *L. starkeyi*, and their co-culture inoculum through the bioremediation of POME. The extraction of lipid was performed by EP technique and the microbial cell disruption was visualized by field emission electron microscopy (FESEM). Furthermore, the fatty acid methyl esters (FAME) composition of lipids accumulated by *B. cereus*, *L. starkeyi*, and their co-culture in POME was characterized using gas chromatography and mass spectrophotometry (GC-MS).

The lipid production performance and bioremediation efficiency (COD removal) were optimized using RSM to maximize the performance of the targeted co-culture

inoculum. The operational parameters (inoculum composition, initial pH, incubation temperature, and time) were considered as independent variables while the COD removal efficiency and lipid production were dependent variables.

1.5 Justification of the Study

In this study, a combined approach has been attempted for the first time to bioremediate POME wastewater and concurrently synthesize the microbial lipids using a pure culture of *B. cereus, L. starkeyi,* and their co-culture. In designing co-culture inoculum, *B. cereus* was selected due to its capability of utilizing a broader range of substrates including real wastewater, especially POME, while *L. starkeyi* was preferred as a robust lipid producer. Furthermore, *B. cereus* can survive in the harsh environmental conditions (i.e., low pH, high temperature) due to their spore forming capability and *L. starkeyi* can degrade oily substrate like POME by excreting several enzymes such as lipases. In addition, a novel electroporation technique was applied to extract lipid from microbes and the lipid extraction yield was compared with several typical extraction methods to justify the efficiency. Finally, the optimization of the operational conditions such as pH, temperature, cultivation period, and inoculum compositions were carried out using central composite statistical design (CCD) to maximize the lipid production as well as COD removal efficiency. To the best of our knowledge, no study has reported the potential of a pure bacterium, especially *B. cereus*, a yeast like *L. starkeyi* and their co-culture to accumulate lipids through the bioremediation of POME. Thus, this approach of microbial lipid production and simultaneous bioremediation of POME can be considered as a novel study combining renewable energy production and environmental resilience.

1.6 Organization of the Thesis

The first part of the thesis presents the background and importance of accumulating microbial lipid through bioremediation using a pure culture of *B. cereus, L. starkeyi,* and their co-culture. The second part of the thesis provides a detailed literature review and methodology, while the final part presents the results and discussions. Chapter 1 mainly focuses on the background of the study, along with the study objectives; Chapter 2 describes the past research efforts related to POME treatment, microbial lipid synthesis,

and lipid extraction methods. The effect of microbial community composition on the performance of lipid accumulation and POME remediation is also discussed. Chapter 3 discusses the materials and methods used in this research. The sample collection and culture medium preparation, inoculum preparation, determination of cell concentration and growth kinetics study, wastewater treatment analysis, biomass harvesting and lipid extraction, effect of different extraction methods are discussed. This chapter also provides the detailed information regarding the optimization of several parameters using co-culture inoculum, experimental design and data analysis for optimization and statistical analysis. Chapter 4, 5, 6 and 7 describe the significant findings of this study, including the bioremediation efficiency of yeast-bacteria co-culture, efficiency of EP for microbial lipid extraction, lipid accumulation performances using different inoculum, effect of different process parameters on the performance of COD removal efficiency and lipid accumulation, and optimization of the performance. In addition, Chapter 8 presents the conclusion derived from the study and the recommendations for future studies.

CHAPTER 2

LITERATURE REVIEW

2.1 Palm oil mill treatment and bioenergy production

2.1.1 Palm oil mill effluent (POME)

The wastewater, POME is one of the major pollutants in Southeast Asia. Specifically, the production of palm oil is abundant in Indonesia, Malaysia, and Thailand. Approximately, there were around 94.76 and 60.88 million tonnes of POME produced in the year of 2015, by Indonesia and Malaysia, respectively (Choong et al., 2018). Moreover, the production of each tonne of crude palm oil usually results in the production of at least 2.5 ton of POME (Chan & Chong, 2019). Thus, Malaysia is estimated to be producing around 80 million tons of POME annually (Taha & Ibrahim, 2014). In Malaysia, the palm oil industry has grown by leaps and bounds over the last five decades and the number of palm oil mills was 454 with an estimated palm oil production of 19.9 million tonne in 2017 (Chan & Chong, 2019; Chin et al., 2013). According to Malaysian Palm Oil Board, Malaysia recorded 19.5 million tonnes in palm oil production in 2018. The production had last pegged 2019 output at 20 million tonnes and is forecasted to rise to 20.3 million tonnes this year (Akhbari et al., 2019; Chan & Chong, 2019).

POME is the most problematic pollutant from the palm oil industry due to its high load of organic contents. POME contains a mixture of wastewater from clarification (60%), sterilization (36%) and hydro-cyclone (4%) units (Akhbari et al., 2019). Raw POME is a colloidal matter which contains 95-96% water, 0.6-0.7% oil and 4-5% total solids containing 2-4% suspended solids. The suspended solids are mostly from palm fruit mesocarp (1) sterilizer condensate, (2) sludge separator and (3) hydro-cyclone waste (Ohimain & Izah, 2017). Several suspended components includes cell walls, organelles, short fibers, and carbohydrates spectrum ranging from hemicellulose to simple sugars, a range of nitrogenous compounds from proteins to amino acids, free

organic acids and an assembly of minor organic and mineral constituents (Ahmad et al., 2016).

Table 2.1 Typical characteristics of POME.

Parameter	**Average (mg/L)**	**Metal**	**Average (mg/L)**
pH	4.7	Phosphorus	180
Oil and grease	4,000	Potassium	2,270
Biochemical oxygen demand (BOD)	25,000	Magnesium	615
Chemical oxygen demand (COD)	50,000	Calcium	439
Total solids (TS)	40,500	Boron	7.6
Suspended solids (SS)	18,000	Iron	46.5
Total volatile solids (TSS)	34,000	Manganese	2.0
Ammoniacal nitrogen (AN)	35	Copper	0.89
Total nitrogen (TN)	750	Zinc	2.3

Source: Ahmad et al. (2016)

Palm oil industry produces huge pollution load into the rivers, due to its high chemical and biological oxygen demand of POME as shown in Table 2.1. Many palm oils mills have not adhered to the wastewater discharge limits. If every human being is supposed to produce 14.6 kg BOD yearly, the BOD of POME is equal to the waste produced by 75 million populations which is around thrice the existing population in Malaysia (Ahmad & Chan, 2009). POME is considered as one of the major sources of pollution load in the water bodies when discharge does not meet the requirements of the Department of Environment Malaysia (Ding et al., 2016).

2.1.2 Palm oil mill effluent treatment

POME is a viscous brown liquid with fine suspended solids at 4-5 pH (Ohimain & Izah, 2017). Naturally POME contains high organic content mainly composed of oil and fatty acids, carbohydrates (29.55%), proteins (12.75%), nitrogenous compounds and a significant amount of cellulose and non-toxic minerals, which can be a good source for microbial fermentation (Sangeetha et al., 2016). POME contains high chemical and biological oxygen demand and oil and grease, due to the lignocelluloses and hemicelluloses components of the material. This can cause considerable environmental problems if discharged without proper treatment. The characteristics and the parameter limits for POME discharge into water courses in Malaysia are summarized in Table 2.2.

Table 2.2 Characteristics and parameter limits for POME discharge into water courses in Malaysia.

Parameter	**Mean (mg/L)**	**Range (mg/L)**	**Limits of discharge (mg/L)**
pH	4.2	3.4-5.2	5.0-9.0
Temperature	-	80-90	45
Biochemical oxygen demand	25,000	10,250-43,750	100
Chemical oxygen demand	51,000	15,000-100,000	-
Total solids	40,000	11,500-79,000	-
Suspended solids	18,000	5,000-54,000	400
Volatile solids	34,000	9,000-72,000	-
Oil and grease	6,000	130-18,000	50
Ammoniacal nitrogen	35	4-80	150
Total nitrogen	750	180-1400	200

Note: Units in mg/L except pH and Temperature (°C); the sample for BOD analysis is incubated at 30°C for 3 d.

Source: Ahmad et al. (2016)

Various treatments methods have been used to treat POME including: (a) anaerobic/facultative ponds; (b) tank digestion and mechanical aeration; (c) tank digestion and facultative ponds; (d) decanter and facultative ponds; (e) physico-chemical and biological treatment; (f) membrane treatment; (g) evaporation and clarification pond coupled with filtration and aeration (Ahmad et al., 2016). The conventional treatment is the pond system and biological treatment. In most mills, the under-sized biological treatment system is unable to cope with increasing POME volume (Ahmad et al., 2016). Cost effective treatment is needed to ensure sustainable economic growth of oil palm industry whilst protecting the environment. Anaerobic treatment of POME is widely used because of its low operational cost. During anaerobic treatment, a large amount of biogas, a mixture of colorless flammable gases, are produced. Nevertheless, there are more than 85% of palm oil mills in Malaysia treating POME by using the low-cost ponding system (Choong et al., 2018). There are several disadvantages associated with conventional POME treatment such as long hydraulic retention times (HRT), low treatment effectiveness, more sludge production, wide land area requirement. Besides the disadvantage of large area required, the escaping greenhouse gases (CHGs, such as CO_2 and CH_4) to the atmosphere is another serious environmental problem of this treatment system. In fact, every tonne of POME treated in the anaerobic ponding system will generate 12.36 kg of methane gas (Choong et al., 2018). Obviously, the POME management system critically needs to be switched to the more sustainable treatment practice for the cleaner palm oil industry development. Table 2.3 shows different treatment systems used for POME treatment.

Table 2.3 Treatment methods of POME with comparison of economical, environmental and operational aspects.

Treatment method	Example	Economical Aspect		Environmental Aspect		Operational Aspect	
		Pros	**Cons**	**Pros**	**Cons**	**Pros**	**Cons**
Anaerobic digestion	Rotating biological contactor Activated sludge reactor		Very energy intensive		Inefficient treatment		Requires incorporation of other treatment systems
Anaerobic ponding system		Low cost			Large area required Uncaptured GHGs	Reliable and stable Simple	Long HRT
Anaerobic digestion high rate closed system	Continuous stirred tank reactor Up flow anaerobic sludge blanket reactor Anaerobic fluidized bed reactor		High cost	Captured GHGs Higher treatment effectiveness			Relatively more sophisticated
Chemical treatment	Coagulation and flocculation Flotation Adsorption		Extra cost required		Inefficient treatment		Pretreatment of POME required
Physical treatment	Sedimentation Centrifugation	Low cost			Inefficient treatment		Pretreatment of POME required

Generally, ponding-based anaerobic and aerobic digestion are the most commonly used techniques for POME treatment. The ponding system has low maintenance costs due to operational and process simplicity; therefore, it is economical and considered to be a feasible means of treating high strength organic wastewater (Ismail et al., 2013; Lek et al., 2018). However, it entails a long hydraulic retention time (generally 40 to 200 days) and a large land area (approximately 1 to 5 ha). Moreover, it is not considered environmentally friendly because the methane produced by this technique is wastefully released into the atmosphere, and the system cannot be certified for carbon emission reduction trading (Ismail et al., 2013; Lek et al., 2018). In some cases, POME was subjected to physical and chemical pretreatment consisting of coagulation and flocculation aid, sedimentation, and adsorption using activated carbon or membrane filtration (Liew et al., 2015). However, the applicability of a wastewater processing system associated with a higher cost might be unpersuasive to the palm oil millers. Therefore, economically viable and environmentally friendly treatment methods should be developed by valorizing POME. It has been presented in some recent reports that POME contains a significant amount of nutrients such as carbohydrates, proteins, vitamins and mineral salts which can stimulate the growth of heterotrophic microorganisms (bacteria, yeast, fungi, and microalgae) (Bala et al., 2015; Gobi & Vadivelu, 2013). Moreover, the highly concentrated nitrogenous compounds in POME could be beneficial for the cultivation of various types of microorganisms (Bala et al., 2015). Oleaginous microorganisms are capable of converting several waste substrates like POME into lipids (Pirozzi et al., 2014). Therefore, POME can be treated by oleaginous microorganisms to produce microbial lipids that could be a potential substitute for plant oils, as a future source of biodiesel (Cheirsilp & Louhasakul, 2013) as well as the bioremediation of POME.

2.1.3 Bioremediation of wastewater and biodiesel

Generation of biogas is a significant part of the biogeochemical carbon cycle. Methanogens (methane producing bacteria) are the last link in a chain of micro-organisms which decompose organic material and return the decomposition products to the environment. Other than that, since early days, microalgae are being used for human health food products, feeds for fish and livestock, and cultured for their high value of oils, chemicals, pharmaceutical products, and pigments. Microalgae can produce varied forms

of renewable biofuels including biomethane, biodiesel, bioethanol, and photobiologically produced biohydrogen (Suganya et al., 2016). The concept of biofuel production from microalgal biomass is not ideal, but getting more attention due to increasing petroleum prices and more widely, the concern about global warming and climate change from the burning of fossil fuels (Shuba & Kifle, 2018). Microalgae can use solar energy for biomass production with relatively less land requirement than conventional agriculture. Figure 2.1 shows a conceptual model of biofuel production from algae.

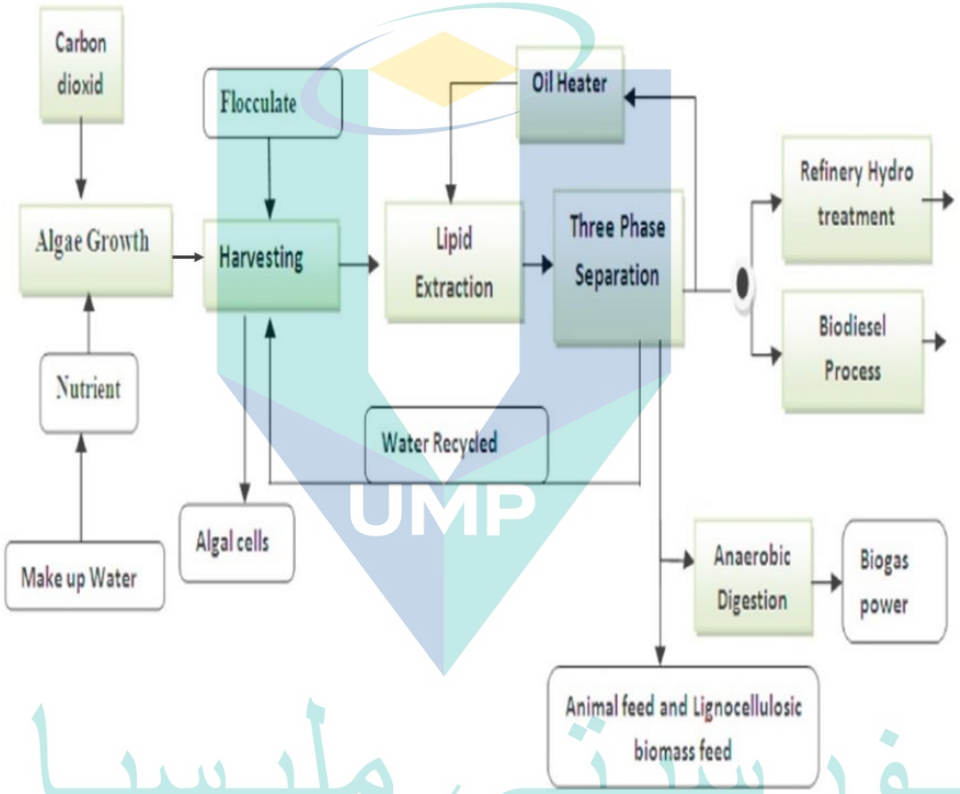

Figure 2.1 A conceptual model for integrated microalgal biomass and biofuel production.

Source: Ahmad et al. (2016)

Among earliest research into the development of microbial biofuels, National Renewable Energy Laboratory has been the pioneers from 1978 to 1996. It concludes that a more practical method for the microbial biodiesel production is to utilize wastewater treatment for cultivation, an already well-developed technology (Ahmad et al., 2016). Oleaginous microbes like algae or yeast readily take up heavy metals like cadmium from the environment and then make a heavy metal stress response, which includes production of heavy metal binding factors and proteins. Several macroalgal species such as *Lamiaria,*

Sargassum, Macrocystis, Ecklonia, Ulva, Lessonia, and Durvillaea have been reported for binding of heavy metals such as copper, nickel, lead, zinc and cadmium (Manzoor et al., 2019). Higher heavy metal levels may hinder other main processes (e.g. photosynthesis and growth) and finally may kill the cells. Treatments of industrial, municipal and agricultural wastewaters by microalgae culture systems have been reported (Samorì et al., 2013; Zhu et al., 2013). Algae enhance degradation; improve CO_2 balance and lower energy demand for oxygen supply in aerobic treatment stages. The algae have a dual role of incorporating nutrients and to provide oxygen to bacteria. Bacteria, in turn, involve in the degradation of organic material in wastewater, the same process utilized in activated sludge. Cyanobacteria and *Chlorella* sp. are reported to be efficient for the treatment of organic pollutants from paper industry wastewater (Pandey et al., 2019).

Besides, oleaginous microorganisms are capable of reducing the COD and BOD in wastewater and a more economical way to eliminate pathogens, phosphorus, and nitrogen than activated sludge (Mujtaba et al., 2018). Cheah et al. (2018b) reported that the microalgae *C. sorokiniana* produce a higher biomass (2.12 g/L) and lipid yields (11.21%) in pretreated POME and simultaneous pollutants removal efficiencies were 62.07% (TN), 47.09% (COD) and 30.77% (TP). Louhasakul et al. (2016) valorized POME into lipid (1.64 ± 0.03 g/L) and lipase (3353 ± 27 U/L) by marine yeast *Yarrowia lipolytica* and evaluated their potential application in biodiesel production. The yeast *Galactomyces reessii* was cultured in POME, and the activity of the ligninolytic enzymes (laccase and manganese peroxidase) and phenol removal was demonstrated by Chaijak et al. (2018). In another study, Ganapathy et al. (2019) studied the boremediation POME using *Meyerozyma guilliermondii* which resulted in a substantial reduction of COD of 72%, total nitrogen of 49.2% removal, ammoniacal nitrogen of 45.1%, total organic carbon of 46.6%, and 92.4% removal of oil and grease after 7 days of treatment period.

2.1.4 Waste substrates for biodiesel production

SCOs produced by oleaginous microorganisms is the supplementary source of conventional oil and fat. The economic values of these bioprocess has become more favorable when zero or negative value waste substrates are utilized as carbon or nitrogen sources (Madani et al., 2017). Glucose is the most common and suitable substrate used in lipid production process because most oleaginous microorganisms are capable of

assimilating glucose for producing SCO. Although it is the best choice for evaluation of lipid production in oleaginous micro-organisms (Fakas et al., 2009), but the high cost of biodiesel is the major obstacle for its commercialization. Using of waste oil and other cheap raw materials can reduce the cost of process (Fei et al., 2011; Karatay & Dönmez, 2010).

Abundance and low cost of lignocellulosic compounds is very important from economical point of view (Madani et al., 2017). Lignocellulosic materials are good substrates for microbial oil production because of the abundance and low cost (Khot et al., 2012; Zheng et al., 2012). Hemicellulose is the main component of lignocellulosic materials; also it is the second polysaccharide in the nature and a good source of xylose. The most abundant monomers in hemicellulose are xylose compounds, so identification of yeasts and bacteria with the ability of assimilating xylose and converting it to desired product is valuable (Madani et al., 2017). Some of oleaginous yeasts and bacteria can metabolize pentoses. This shows the ability of TAG production from lignocelluloses substrates and other low cost materials (Sabirova et al., 2011; Zheng et al., 2012). Pan et al. (2009) isolated oleaginous yeasts with assimilating capacity of xylose and the best yeast strain could produce 5.8 g/L lipid while using 40 g/L xylose. Dai et al. (2007) used environmental residues such as corn stalk and rice straw as sole carbon sources for lipid production using yeast like *Rhodotorula glutinis*. The results for lipid content were 11.78 and 5.74% on corn stalk and rice straw, respectively. Enshaeieh et al. (2018) used *R. mucilaginosa* on xylose, wheat straw, rice bran, grass hydrolysate and leaves hydrolysate and obtained lipid yield of 8.1, 5.9, 6.2, 7.5 and 6.8 g/L, respectively. Enshaeieh et al. (2012) reported lipid yield of 7.12 and 6.54 g/L for *R. aurantiaca* on rice bran and wheat straw, respectively.

2.1.5 Wastewater as a source of microbial lipid for biodiesel production

Recently, the use of industrial wastewater as a feed stock to produce microbial lipids for non-fossil biofuels (i.e., biodiesel) production is considered as a sustainable solution as it simultaneously addresses the need for low cost growth medium for cultivating microbes and the reduction of the environmental burden posed by wastewater. A variety of organic wastes, especially, industrial wastewater like palm oil mill effluent (POME) (Bala et al., 2015; Karim et al., 2019; Louhasakul et al., 2016), citrus wastewater (Karim et al., 2018b; Qadeer et al., 2018), dairy waste effluent (Gawai et al., 2017; Kumar

et al., 2015), milk processing wastewater (Cea et al., 2015), apple waste (Qadeer et al., 2018) have been widely used in the microbial lipid production. It has been reported in several recent studies that the wastewater like POME contains a significant amount of nutrients such as carbohydrates, proteins and mineral salts which can stimulate the growth of heterotrophic microorganisms including bacteria, yeast, fungi, and microalgae (Bala et al., 2015; Islam et al., 2018c; Karim et al., 2019). Although various strains of several oleaginous microbes could accumulate significant amount of lipids but not all of them may not be able to efficiently utilize the nutrients from wastewaters like POME. However, certain microorganism native to special environments may be more prone to produce cellular lipids by assimilating the nutrients from POME.

2.2 Cell disruption and lipid extraction techniques for potential biofuel production

2.2.1 Overview of cell disruption

Biofuels from microbial lipids are considered as one of the most promising substitutes to the non-renewable fossil fuels. However, the efficient recovery of microbial lipids is emergent for the overall process economics and sustainability. The conventional lipid extraction technique entails direct use of organic solvents to liberate the intracellular lipids, thus making the extraction process more time-consuming and less eco-friendly. Furthermore, the efficiency of extraction process is affected by several problems, such as the rigid structure and composition of microbials cell walls, the water content of biomass, the limited accessibility of lipids, the reduced mass transfer, the formation of stable emulsions, etc. Various modifications (e.g., use of green solvents, direct transesterification, pretreatment for disrupting microbial cells, etc.) to conventional lipid extraction have been proposed to mitigate the problems associated with lipid extraction. Among them, the pretreatment approach (i.e., mechanical, chemical, biological cell disruption) in combination with solvent based extraction would be a promising approach to address the problems during lipid extraction. Therefore, intensive research, review and analysis are required to understand the challenges in lipid extraction and to develop a useful extraction process in terms of biomass status (dry/wet), solvent use, lipid yield, extraction time, and scalability. In this chapter, we have reviewed the composition and possible applications of microbial lipids with the main challenges in their extraction

process. Furthermore, the advantages and limitations of several pretreatment methods for cell disruption have been comprehensively discussed.

2.2.2 Importance of cell disruption in microbial lipid extraction

The conventional fossil fuels such as petrol, diesel, coal, natural gas, etc. are considered as the basic sources to meet this energy demand (Yousuf et al., 2017c). However, the progressive depletion of these petroleum-based fuels is recognized as a future challenge. In this context, the concern regarding alternative sources of energy to replace the fossil fuels is increasing tremendously (Alhattab et al., 2019; Yousuf et al., 2017c). The increasing demand for bioenergy sources and bioactive compounds has intensified research into biofuels as a viable renewable source to fulfil these needs. The biofuels (e.g., biochar, biogas, biohydrogen, biodiesel, bioethanol, etc.) produced from biomass (e.g., wood and wood residues, plants, animal matters, waste energy feedstocks, microbial biomass, etc.) have been considered as sustainable renewable sources to meet the future energy demand (Bharte & Desai, 2018; Karim et al., 2018b). Among them, microbes-derived biodiesel has gained widespread interest as one of the promising substitutes to the non-renewable fossil fuels.

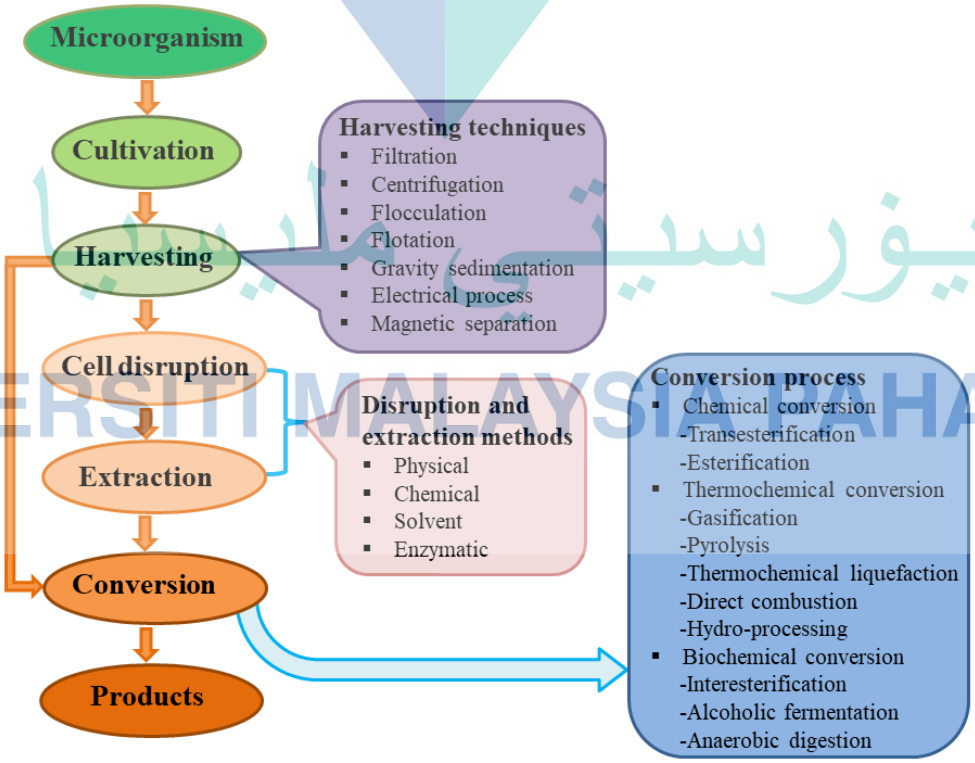

Figure 2.2 Key route of the oleaginous microbes processing to obtain different valuable products.

Oleaginous microorganisms produce a wide range of valuable nutrients useful in various industries, such as proteins and carbohydrates, along with the lipids used to produce biofuels (Chew et al., 2017). For example, the microbial lipid yield were estimated to be 20,000-80,000 L/acre/year, which are about 30 times more than those obtained from seed crops fuels (Enamala et al., 2018). In addition, oleaginous microbes are considered as effective source of biodiesel due to the rapid growth rate; capable to grow in various complex environments including wastewaters; high biomass productivity; low land use; non-toxic; biodegradable; less harmful gas emissions (Arenas et al., 2017); diverse biochemical composition (Papazi et al., 2012); and limited competition with the edible crops; etc. (Bharte & Desai, 2018). However, large quantities of chemicals or high energy inputs are required for the extraction of intracellular compounds (Figure 2.2) due to the recalcitrance, complexity, and diversity of microbial cell walls (Gerken et al., 2013). Consequently, the use of oleaginous microbes as a feedstock for biofuel production is hindered by the process economics and sustainability (Alhattab et al., 2019). For instance, about 25-75% of the microbial biomass comprises stored lipids, however, extraction of lipids is the most challenging and energy intensive procedure due to the tiny microbial cells, rigid cell walls, and limited contact between the solvents during lipid extraction (Bharte & Desai, 2018).

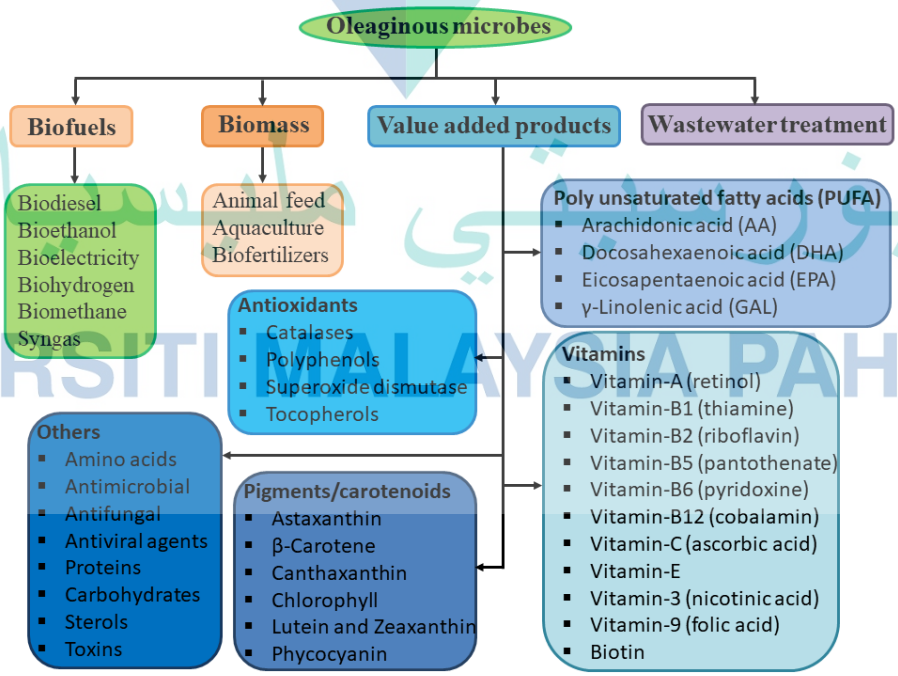

Figure 2.3 The potential applications of microbes and different valuable products.

Generally, the cell walls of microorganisms are structurally robust, complex, and chemically diverse and therefore, the disruption of the microbial cell wall is the most crucial step to extract different valuable biomolecules (Figure 2.3) from the cells. Moreover, the lipid extraction process is often influenced by the water content of biomass, selection of suitable solvents, the blocking effects from insoluble biomass residues, the limited lipid accessibility, and the formation of stable emulsions, etc. Therefore, several alternative approaches (e.g., use of green solvents, direct transesterification, pretreatment for disrupting microbial cells, etc.) for lipid extraction have been proposed to mitigate the problems associated with conventional lipid extraction process. Among them, the pretreatment approach in conjunction with solvent based extraction can be an emerging approach to address the problems during the extraction of microbial lipids. Recently, the extraction processes are often conducted with solvents using untreated, chemically treated or mechanically treated cells. These methods of treating microbial cell to enhance intracellular lipid extraction are often called pretreatment methods or cell disruption techniques (Alhattab et al., 2019). Various cell disruption techniques (e.g., physical, chemical, and biological methods, etc.) have been reported to extract the desired compounds from microbial biomass (Bharte & Desai, 2018; Brennan & Owende, 2010; Dong et al., 2016). The physical techniques include bead beating, high-pressure homogenisation, ball milling, microwave heating (Iqbal & Theegala, 2013), ultrasonication, hydrodynamic cavitation, thermolysis, electrocoagulation, osmotic shocks, electroporation and laser treatments (Sati et al., 2019), whereas the chemical methods are based on selective interaction of the cell walls with certain chemicals, such as, chloroform; methanol; hexane; isopropanol; acetone; dichloromethane; and so on. Some other methods include autoclaving and lyophilisation (Lee et al., 2010), supercritical carbon dioxide extraction (Crampon et al., 2013), etc. However, there is no agreed conclusion of the most suitable pretreatment method for different algal species. Therefore, in this chapter, the challenges of lipid extraction and the proposed alternatives to overcome the challenges for enhanced product recovery are discussed. The importance of a suitable cell disruption technique for enhanced product recovery is also focused in this regard.

2.2.3 Microbial lipid composition and distribution

Oleaginous microorganisms are gaining increased interest compared to other sources (Borowitzka, 2013; Mehta et al., 2018), since they can accumulate diverse kinds of value-added components (e.g., proteins, lipids, carbohydrates and pigments) (Figure 2.3). Generally, oleaginous microorganisms contain a wide range of lipid classes (Figure 2.4), such as free fatty acids (FFAs), glycolipids, phospholipids, acyl-glycerides, lipoprotein, hydrocarbons, sterols, and pigments. The lipid classes have different physical and chemical properties, like solubility, polarity, viscosity, etc. (Enamala et al., 2018). The lipids can be classified as polar and nonpolar, based on their polarity and chemical structure of molecular head group. The polar lipids of microbes are used to form the cell membranes and include glycolipids and phospholipids. On the other hand, the nonpolar lipids (i.e., neutral lipids) are usually used as the energy source and comprised of FFAs and acylglycerols (mono, di, and tri) (Subhash et al., 2017). The key ingredients of both polar and nonpolar lipid molecules are the long chain (comprising 12-22 carbon in length) fatty acids, which can be unsaturated (at least one double bond) or saturated (no double bonds). It is worthy to note that the quality and production of biodiesel is directly affected by the composition of fatty acids (Sati et al., 2019). However, triacylglycerols (TAGs) are the main target to produce biodiesel among the different lipid groups, because of their lower degree of unsaturation compared to other lipid fractions.

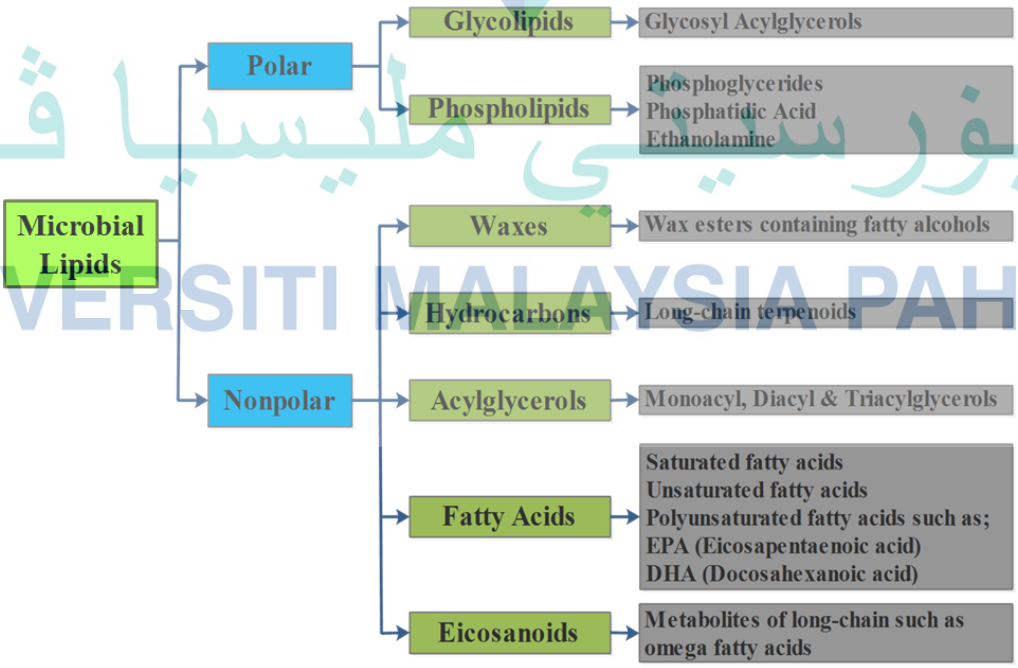

Figure 2.4 Different classes of microbial lipids with examples.

Generally, lipids are stored in different locations in the cell and play a significant role since the have specific cellular functions. The lipid bodies (containing mostly of sterol esters and TAGs) are surrounded by a phospholipid monolayer and exist in the cytoplasm as a form of energy storage. These lipid-rich cell compartments are found in all eukaryotic organisms, and also in some prokaryotic genera, e.g., *Streptomyces* and *Rhodococcus* (Dong et al., 2016). However, the rigidity of microbial cell wall impedes the efficient extraction of lipids for biodiesel production. Eventually, the yield of biodiesel depends on the lipid contents of individual cells, which varies significantly for different microbial species. Biodiesel production from oleaginous microorganisms (such as microalgae, bacteria, yeast), includes several upstream and downstream operations, including cultivation of microbes; biomass harvesting; lipid extraction; and transesterification of the lipids (Figure 2.5). Among them, the extraction of lipids is the most challenging step, and it represents an significant bottleneck for industrial scale biodiesel production (Karim et al., 2018d). Since lipids are generally synthesized in the cellular compartments of the microbial cells in the form of lipid droplets; hence, it is essential to break up the cell walls in order to improve the extraction yield of the intracellular compounds from biomass. Various chemical, mechanical, biological, and physiochemical pretreatment methods could be applied to disintegrate the microbial cellular membranes (Patel et al., 2018). Therefore, a suitable cell disruption technique can be considered as a key factor to improve the lipid extraction efficiency in microbial oil based biodiesel production (Gerken et al., 2013).

2.2.4 Microbial lipid extraction mechanisms

The lipid extraction from microbes is generally carried out from dried biomass (dry route) or wet biomass concentrate (wet route) (Sati et al., 2019). In most cases, lipids are extracted using solvent based techniques, hence solvents play a vital role in both routes. In some cases, solvents are subjected directly into cell pellets while in other cases, solvents are employed during cell disruptions (Alhattab et al., 2019; Bharte & Desai, 2018). Generally, lipids are dissolved into a number of solvents, such as methanol, ethanol, butanol, isopropanol, chloroform, n-hexane, acetone, benzene, and cyclohexane; however, hexane, chloroform and methanol are regarded as the most potential solvents to extract microbial lipids (Brennan & Owende, 2010; Dong et al., 2016). The solvents should have high specificity for intracellular lipids, insoluble in water, greater penetration

ability through the cell matrix, low boiling point, volatile, inexpensive, and non-toxic characteristics in order to be efficient for product recovery (Sati et al., 2019).

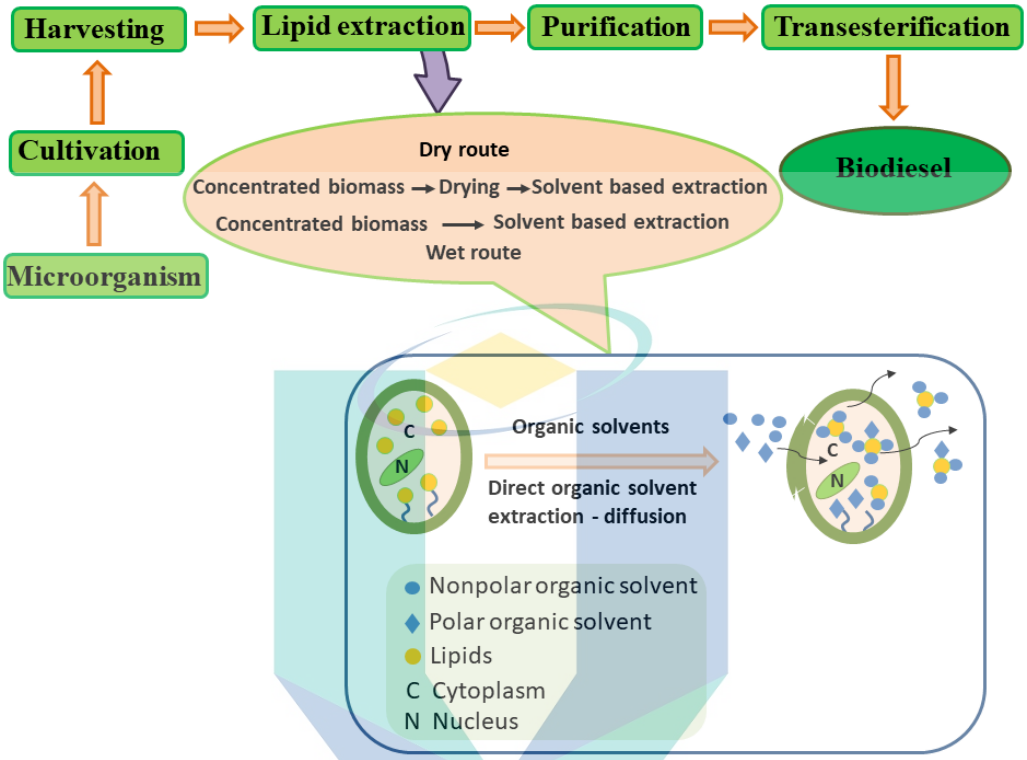

Figure 2.5 Key steps of microbial oil-based biodiesel production process. Source: Modified from Sati et al. (2019)

In solvent extraction, generally, a mixture of polar and nonpolar solvent, with a particular mixing ratio has been used for complete recovery of intracellular lipids (Brennan & Owende, 2010). The solvent molecules penetrate the cell walls, subsequently enter to the cytoplasm, and form the complex structures with neutral lipids (which exist in the cytoplasm in the form of globules) while microbial biomass is subjected to a nonpolar solvent (Figure 2.5). These lipid-solvent complexes diffuse out of the cell walls into the bulk solvent due to variation of concentration gradient (Dong et al., 2016). However, a certain fraction of neutral lipids is remained inside the cell as a complex with polar lipids (which attached to the cell membrane protein via hydrogen bond). Hence, the use of polar solvent is emergent to disperse these lipid fractions. The polar solvents separate these neutral lipids from the complex via formation of hydrogen bonds with the polar lipids (Sati et al., 2019). This process inevitably brings out the polar lipids into the bulk solvent. Finally, they are recovered by the distillation or evaporation of the solvents.

2.2.5 Challenges in microbial lipid extraction process

The rigid cell wall of microbes may prevent direct contact between the solvent and the cell membrane, and ultimately, hinders the lipid extraction. Besides, the physiological properties e.g., the location where the lipid is stored, and the process by which lipid contents accumulated in the cell can also influence the efficiency of the solvent (Brennan & Owende, 2010). Apart from these, lipid recovery from wet biomass in a practical extraction process, can be also affected by other factors such as, water content in cell biomass; selection of the solvents; mass transfer mechanism; lipid accessibility; formation of emulsions; etc. (Dong et al., 2016; Yousuf et al., 2017b). However, these important factors are overlooked since the cell disruption was mostly focused in all studies of wet lipid extraction, even though these factors are crucial to develop an economic and sustainable process.

2.2.6 The role of cell wall structure and composition on product yield

Some microbes such as microalgae or yeast form robust cell walls having a tensile strength of ~9.5 MPa, which about 3 times stronger than the plant cells (Bharte & Desai, 2018). The robust cell wall structure of various species acts as a barrier to the commercial utilization of microbial oil. Generally, cell wall are comprises of cellulose, glycoproteins, protein, uronic acid, xylan, mannose, and minerals such as calcium or silicate (Bharte & Desai, 2018). For example, the complex sugars forming microbial cell walls (e.g., *Tetraselmis striata, Tetraselmis suecica,* etc.) are mainly made of up of galactose, mannose, xylose, rhamnose, and arabinose. The high content of glucose and mannose contributes to the rigidity of cell wall (Alhattab et al., 2019). Consequently, the cell wall structure and composition of microbes have a significant effect in lipid extraction efficiency and performance as well as in cell disruption techniques. Therefore, a deep insight into the role of cell wall structure, composition, and cell size need to be considered for selecting an appropriate pretreatment technique to increase product recovery.

2.2.7 Effect of biomass water content on lipid extraction

Drying of the biomass is emergent step for lipid extraction and trans-esterification from microbial cells. However, the drying of microbial biomass significantly increase the time and cost of the biodiesel production through transesterification (Brennan & Owende, 2010). Moreover, the drying temperature may affect in the composition and amounts of

lipids during the extraction from algal biomass. For instance, drying the biomass at 60-70 ℃, the TAG in lipids remain unchanged and the total lipid content may decrease slightly; however, both the concentration of TAG and lipid yield may decrease with a higher temperature than that of normal range (Brennan & Owende, 2010; Yousuf et al., 2017b). Therefore, the wet biomass have been preferred to minimize the production cost and process difficulties; however, the extraction efficiency of lipids would be decreased if microbial biomass contains high water content (Yousuf et al., 2017b). In a recent study, Ehimen et al. (2010) reported that the oil to fatty acid methyl ester (FAME) conversion was practically hindered with microbial biomass having a water content more than 32%; however, 82% FAME conversion was achieved when water content was reduced to 0.7%. In these cases, a higher proportion of alcohol to total lipid content is required, which may not be eco-friendly. Nevertheless, the extraction of lipids from wet microbial biomass experienced several challenges such as reduced mass transfer, limited accessibility of lipids, and formation of stable emulsions, etc. (Dong et al., 2016). Therefore, intercellular products *i.e.* extraction of lipids with solvents are difficult from wet algal biomass without a suitable pretreatment (Brennan & Owende, 2010).

2.2.8 The solvent extraction

The solvent extraction is usually employed in extraction of lipids from wet biomass in most of the transesterification studies, particularly in direct transesterification. However, heating for reflux needs higher energy consumption and suffers from many difficulties in solvent extraction, hence, the scale-up process is still limited (Park et al., 2015; Yousuf et al., 2017b). Cheirsilp and Louhasakul (2013) observed that the direct transesterification without addition of nonpolar solvent required longer reaction time (~6 h) with a methanol/biomass ration of 125:1. However, the reaction time decreased to 1 h with an increase in methanol/biomass ratio to 209:1. This is due to the higher water content of reaction medium. In another study, Wahlen et al. (2011) reported that wet microbial cells having a water content of greater than 50% required a higher amount of methanol to obtain a FAME content of more than 70%. However, the uses of an excess amount of solvent would not be environmentally friendly; therefore, it is important to overwhelm this limitation. It is presumed that a suitable pretreatment method for cell disruption prior to the extraction could be effective to solve this problem.

2.2.9 Selection of solvent for lipid extraction

In the solvent extraction, a suitable partition coefficient of extraction solvent is imposing the solute to migrate into the solvent phase from the aqueous phase. Therefore, a solvent should be selected so that the lipids have higher partition coefficient in it. However, neutral lipids (i.e., hydrophobic lipids) would be favorably partitioned into the nonpolar solvent phase based on the "like dissolve like" principle; whereas, polar lipids could not been extracted so readily with nonpolar solvents because of their bindings with biomass matrix (Dong et al., 2016; Sati et al., 2019). Thus, the co-solvents are typically used in lab scale extraction to disrupt the linkage between polar lipids and biomass matrix, and to improve the solubility of polar lipids as well.

The efficiency of the solvent extraction is reliant on the selection of solvents and the ratio of the solvents in which these are used. Although, a lots of individual solvents and their combinations have been employed so far; the chloroform: methanol mixture has been reported as a quick, effective, and quantitative combination for extraction of microbial lipid in a number of comparative studies (Bligh & Dyer, 1959; Dong et al., 2016; Folch et al., 1957; Sati et al., 2019). Besides, the chloroform: methanol, in the ration 1:2 (v/v) (widely known as Bligh-Dyer method) (Bligh & Dyer, 1959) and 2:1 (v/v) (commonly known as Folch method) (Folch et al., 1957), are the mostly used mixtures for lab scale studies, having to the high effectiveness. Bligh-Dyer and Folch methods employed methanol and chloroform to increase solubility and accessibility of polar lipids that eventually improve the total yield of lipids (Dong et al., 2016). However, the extraction of lipids in lab-scale studies are mostly conducted in batch mode and limit the lipid extraction once the solvent becomes saturated with the lipid. As a consequent, a continuous organic solvent method (i.e., Soxhlet extraction) is commonly used to overcome this problem.

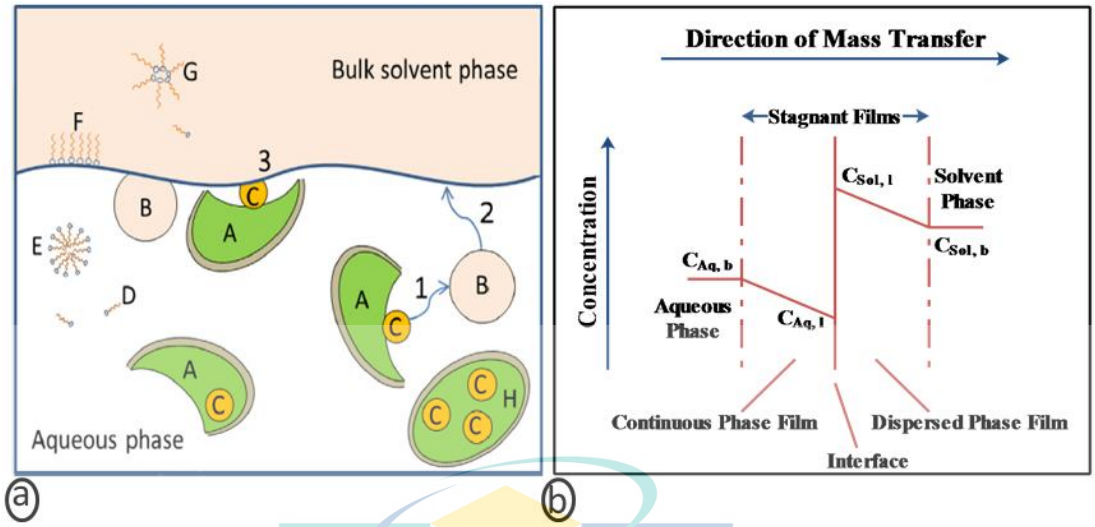

Figure 2.6 a) The mechanism of lipid extraction from wet biomass (A: ruptured cells; B: solvent droplet dispersed in aqueous phase; C: neutral lipids droplet; D: polar lipids; E: polar lipid micelle; F: polar lipids accumulated on the interfacial surface; G: reverse micelle; H: intact microbial cell),. b) The concentration profile for solute transfer from the bulk (*b*) aqueous (*aq*) across the interface (*i*) to the bulk solvent (*sol*) phase based on two film theory.

Source: Modified based on Liddell (1994) and Dong et al. (2016).

Generally, nonpolar lipids (e.g., TAGs and FFAs) exist as the form of small oil droplets in an aqueous environment, since those have reduced solubility in water. These lipids seem to float on the surface of the aqueous phase because of their lighter density. However, these lipid bodies may adhere to or be encapsulated by insoluble cellular debris. Therefore, the mechanism of nonpolar lipid (by using nonpolar solvents) from aqueous environment is to dissolve the tiny lipid droplets into the bulk solvent phase (Figure 2.6a); thereafter, it can be separated from the aqueous biomass residue by phase separation (Dong et al., 2016). In contrast, the mechanism of polar lipid extraction (by using nonpolar solvents) is more critical compared to the nonpolar lipids. The polar lipids are generally ingredients of cell membranes or closely connected with other cellular components and might not be extracted so easily with nonpolar solvents. The polar lipid extraction from an aqueous phase would be imagined by releasing of an ion (or hydrophilic moiety) from its linked water molecule followed by transfer into the solvent. However, the breaking up of the liquid-water interaction needs additional energy to liberate the polar lipids (Liddell, 1994; Walde et al., 1990). Consequently, the extraction of these ionic (or amphiphilic) lipid molecules needs more energy input. In addition, the polar lipids adhere to the solvent-water interface may behave as surfactants and easily lead to emulsion formation through the reduction of interfacial tension (Figure 2.6a). In

conclusion, the co-solvents could be employed to increase the polar lipid recovery as illustrated above; however, they are unlikely to be practical for an industrial scale application due to the complex downstream processing. Alternatively, these lipids could be efficiently released by the biomass pretreatments, such as changing pH or chemical/enzymatic hydrolysis. On the contrary, extraction of the neutral lipids (e.g., TAGs and FFAs) are favored compared to the polar lipids because of their higher partitioning in nonpolar solvents, better solvent recovery, improved mass transfer, and reduced emulsion formation. Therefore, converting the polar lipids into the FFAs by a biomass pretreatment prior to the extraction would be preferred from an engineering perspective.

2.2.10 Limitations of conventional solvent-based extraction and alternatives

The solvent extraction has widely been employed to extract lipids from oleaginous microbes. However, the practical application of conventional solvent extraction is limited due to the huge amount of solvents requirement. In addition, the solvent based lipid extraction methods are time consuming, not eco-friendly and possess low efficiency (Table 2.4) (Dong et al., 2016; Sati et al., 2019). Therefore, intensive research efforts need to be addressed to minimize the problems associated with solvent extraction. Currently, the efforts have been focused in three parallel directions such as finding green solvents (e.g., bio-based solvents, ionic liquids, super critical fluids, switchable solvents, etc.); direct transesterification (i.e., concurrent extraction of lipids and conversion of lipids to biodiesel); and exploring pretreatment approaches of algal biomass (e.g., expeller press, microwave, ultrasound, chemical treatments, enzymatic methods, etc.) (Sati et al., 2019).

Table 2.4 The advantages and limitations of microbial lipid extraction methods.

Methods of extraction	Advantages	Limitations	References
Conventional Solvent extraction	Generally inexpensive, solvents can be recycled, no set-up cost, ease of extraction	Toxic, adverse effect on environment	(Lee et al., 2010; Sati et al., 2019)
Folch method	A standard method of extraction, widely reported to extract lipid	Laborious method, adverse effect of chloroform on the environment	(Enamala et al., 2018)
Bligh and Dyer method	A simple and standard method of extraction, total lipids can be determined, samples can be analyzed directly without pre-drying	Laborious method, adverse effect of chloroform on the environment	(Enamala et al., 2018)
Ionic liquid extraction	Synthetically flexible, thermally stable, non-flammable in a wide temperature range, high conductivity, broad miscibility range, can be recycled, reduced energy consumption, enables solvent extraction	Possible pathway into the environment through wastewater, solvents synthesis is not eco-friendly, higher energy requirement for distillation of solvents	(Choi et al., 2014; Sati et al., 2019)
Supercritical fluid extraction	Quick separation, gives highly purified extracts, low toxicity, highly selective since flexibility of temperature and pressure variations, no separation step is required	Pressure vessel is expensive, high residual water content within microbial biomass is not accepted, because it results in flow impedance and restrictor plugging, high equipment and operational cost (e. g., pressure vessel is expensive)	(Liau et al., 2010; Sati et al., 2019)
Ultrasound assisted extraction	Solvent consumption is reduced, greater penetration of solvents into cell compartment, lower extraction time, higher yield of lipids	Energy intensive, possess scale-up difficulties	(Alhattab et al., 2019; Lee et al., 2010)
Microwave assisted extraction	Simple, easy, highly effective Can be easily scaled-up, higher yield of lipids with superior quality, short duration of extraction time	Energy intensive	(Bharte & Desai, 2018; Lee et al., 2010)
Switchable solvents	Easily recyclable, green approach	Technical viability of the process is yet to be studied; synthesis of solvent can be environmentally damaging	(Sati et al., 2019)
Direct transesterification	Economical process, less energy, reduce time and solvent uses	Paired with organic solvents; hence, it is toxic, less efficient with high water content, thus typical requires dry biomass, technical viability of the process is yet to be studied.	(Sati et al., 2019)

2.2.10.1 Green solvent approach

The uses of green solvents in microbial lipid extraction are of great interest nowadays, with an intention to address the problems raised by organic solvents. As reported in several studies (Kumar et al., 2017; Sati et al., 2019; Tanzi et al., 2012), different kinds of green solvents such as bio-based solvents, ionic liquids, supercritical fluids have been used to replace the organic solvents. Tanzi et al. (2012) reported that the bio-based solvents, derived from agricultural sources such as, Terpenes (e.g., gum terpene, *D*-limonene, and *p*-cymene, etc. produced from citrus species, pine trees) and tree leaf oils could be successfully implemented to extract lipid from *C. vulgaris*. The solvents, derived from citrus, corn, and soybeans e.g., ethyl-lactate, methyl-soyate, ethyl-acetate, 2-methyl tetrahydrofuran, and cyclopentyl-methyl ether have also been regarded as a potential replacement of the organic solvents (Kumar et al., 2017). These solvents were demonstrated to improve the biodiesel quality of the extracted lipids because they resulted in a comparatively low level of polyunsaturated fatty acids (PUFA) in lipid extraction (Mahmood et al., 2017).

Although, the bio-based solvents are biodegradable, non-toxic, and able to replace many hazards; however, the consistent feedstock supply is of a great concern (Sati et al., 2019). Furthermore, the supercritical fluids have been appeared to be an efficient substitute to organic solvents due to safety, health, and environmental concerns. In this technique, the microbial biomass is subjected to the supercritical fluid under controlled conditions of temperature and pressure, then the lipid contents of microbial cells desorbed in the fluid stream which are finally recovered by condensation. CO_2 is widely used in supercritical fluid extraction (SFE) due to its low flammability, lack of reactivity, low toxicity, and recoverable characteristics (Sahena et al., 2009). In a recent study, Tai and Kim (2014) obtained maximum yield of lipids (~ 6.2%) by employing SFE compared to organic solvent-based lipids yield (< 5%). Other than that of bio-based solvents and supercritical fluids, the ionic liquids (i.e., non-aqueous salt solutions composed of an organic cation and a polyatomic inorganic anion) and the switchable solvents (a sub-class of ionic liquids) have also been considered as green solvents (Sati et al., 2019). In a recent study, Kim et al. (2013) obtained 1.6-fold higher lipid yield by using ionic liquid namely, $[Bmim][MeSO_4]$ to extract lipids from *C. vulgaris* with the assistance of ultrasonic pretreatment.

Therefore, it can be supposed that the direct use of green solvents is desirable but still an active area of research and development. As mentioned above, green solvents have been employed in various studies to replace the organic solvents; however, they used them in combination with a pretreatment method to enhance the efficiency of lipid extraction.

2.2.10.2 Direct transesterification

Direct transesterification of microbial lipids have been reported as an easy, simple, and rapid approach for quantifying fatty acids by integrating the extraction and transesterification into a single step (Figure 2.7); also termed as '*in-situ* transesterification' (Yousuf et al., 2017b). This technique entails a solvent-mediated extraction of microbial lipids followed by solvent evaporation and, thereafter, production of FAME (Sati et al., 2019). In this technique, wet or dry biomass is treated with a mixture of methanol and inorganic acid or base catalyst in a single reactor resulting in the reactive extraction of lipids as FAAEs (i.e., fatty acid alkyl esters), typically, FAME. Methanol acts both as an extraction solvent and an esterification reagent (Park et al., 2015). The process of simultaneous lipid extraction and transesterification of lipids to FAME, not only saves the time but also reduces the addition of organic solvents in large amounts. Moreover, this process can decrease the cost of instrument installation and maintenance, and the energy consumption (Sati et al., 2019; Yousuf et al., 2017b). In addition to that there are enough reports where direct transesterification process has been found to be advantageous due to the higher FAME yield compared to two-step processes. For instance, Vicente et al. (Vicente et al., 2009) achieved a higher yield of FAME (>99% of FAME); whereas, the conventional processes produced 91.4-98% of FAME. This is attributed to the involvement of FFAs, phospholipids, and glycerides resulted in higher FAME yield in the *in-situ* transesterification process (Park et al., 2015). Furthermore, this technique has been proved to be efficient in forming biodiesel from both the mono- and mixed cultures of oleaginous microbes (Park et al., 2015; Vicente et al., 2009).

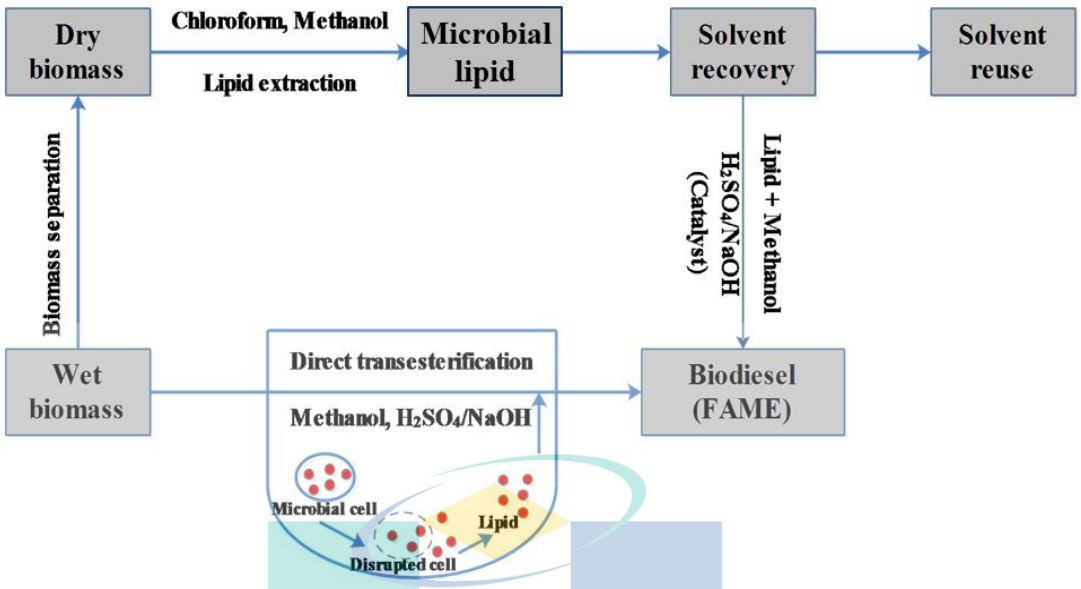

Figure 2.7 The difference between traditional and direct transesterification process. Source: Modified from Yousuf et al. (2017b)

Although the direct transesterification process offers a shorter processing time and lower cost of production, it requires further study to improve the factors that affect the efficacy of biodiesel production from microbial biomass. The major hurdle that must be faced in direct transesterification, is the disruption of microbial cell wall to increase the release of intracellular lipids (Kakkad et al., 2015; Yousuf et al., 2017b). Another major challenge in this process is selection of the catalysts. Usually, homogeneous base catalysts have been used in transesterification reaction due to their moderate reaction conditions and faster reaction rate than the acid catalysts (generally, acid catalyzed reactions need higher temperature and longer reaction time) (Yousuf et al., 2017b). Nevertheless, acid-catalyzed esterification reactions of FFAs results in the water formation, which limits the completion of the reaction. Base-catalyzed reactions, on the other hand, cause soaps formation from cellular FFAs (Kakkad et al., 2015). Furthermore, the water level of the biomass significantly effects the production cost of biodiesel in direct transesterification process. The lipid extraction efficiency is decreased if biomass contains high amounts of water. In addition, this technique has been developed mainly for laboratory scale reactions and is used for analytical quantification of lipids. Generally, most of the laboratory methods use excess amounts of solvents and prolonged reaction time to ensure complete recovery (Yousuf et al., 2017b). Therefore, the method needs to be further modified and optimized for industrial scale applications.

2.2.10.3 Pretreatment methods for microbial cell disruption

The pretreatment approach of microbial biomass aims at disruption of algal cells by using chemical, mechanical, or biological techniques to obtain better product yield. Disruption of microalgal cells is often required to improve intracellular product release from cell compartments because the rigid cell walls and membranes can reduce the extraction efficiency as they are reducing biodegradability of the cells (Alhattab et al., 2019; Bharte & Desai, 2018). Therefore, in most cases, cell disruption prior to lipid extraction is considered as an essential step to eradicate or weaken the protective cell walls of algal cells to make the intracellular lipids more accessible in solvent extraction in order to enhance the lipid extraction yield.

Effects of biomass pretreatment on lipid extraction

A suitable biomass pretreatment method or cell disruption technique could mitigate the problems associated with the lipid extraction process to increase extraction efficiency (Figure 2.8). In algal based biofuel production process, the cell disruption prior to lipid extraction is not only necessary to break-up the cell walls exposing lipids (Islam et al., 2018c; Karim et al., 2018d), but it would also help to liberate combined lipids for better extraction, reduce insoluble solid residues to enhance mass transfer, increase lipid accessibility, and decrease the formation of stable emulsions to improve solvent recovery (i.e., ease phase separation) (Halim et al., 2014; Schwenzfeier et al., 2013). In this way, the amphiphilic polar lipids could be converted into the hydrophobic FFA for a better extraction, and utilized as preferred biofuel precursors with reduced toxicity in a downstream catalytic upgrading (Dong et al., 2016). Therefore, future research for biomass pretreatment should be comprehensively assessed regarding the lipid extraction efficiency, scalability, energy consumption, and compactivity with downstream processing.

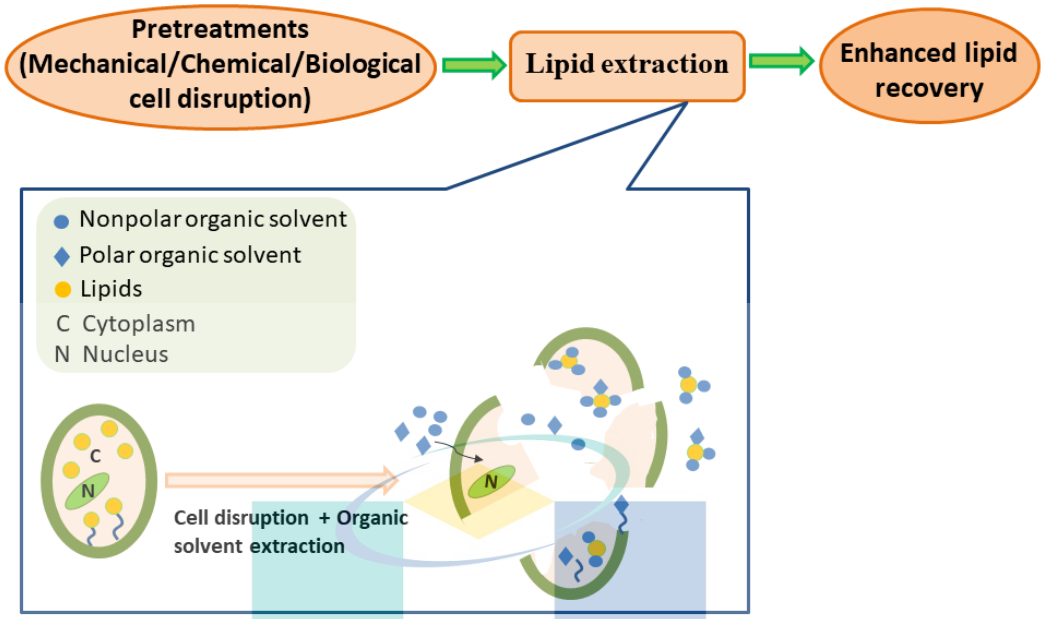

Figure 2.8 Enhanced lipid extraction through complete cell disruption by pretreatment.

Different pretreatment techniques

Various physical, chemical and enzymatic methods have been reported to disrupt the microbial cell walls (Figure 2.9) (Lee et al., 2017). There are two common terms- 'cell wall disruption' and 'cell disintegration'. The cell wall disruption implies to disrupt outer cell wall structure rather inner cellular organizations. However, the cell disintegration entails the rupturing the entire cell, where the cells are no longer recognized as intact cells under microscope. In general, both methods can be used to release the bioactive compounds embedded within the cells. It is demonstrated that the cell wall disruption and cell disintegration could be performed by either mechanical or non-mechanical treatments (Günerken et al., 2015). The mechanical methods includes different physical (e.g., ultrasonication, osmotic shock, microwave, etc.), mechanical (e.g., ball milling, bead beating, high-pressure homogenizer, etc.) or thermal (e.g., autoclave, thermolysis, steam explosion, etc.); whereas, the chemical methods are based on selective interaction of the cell walls with certain chemicals such as chloroform, methanol, hexane, isopropanol, acetone and dichloromethane, etc. (Bharte & Desai, 2018; Onumaegbu et al., 2018).

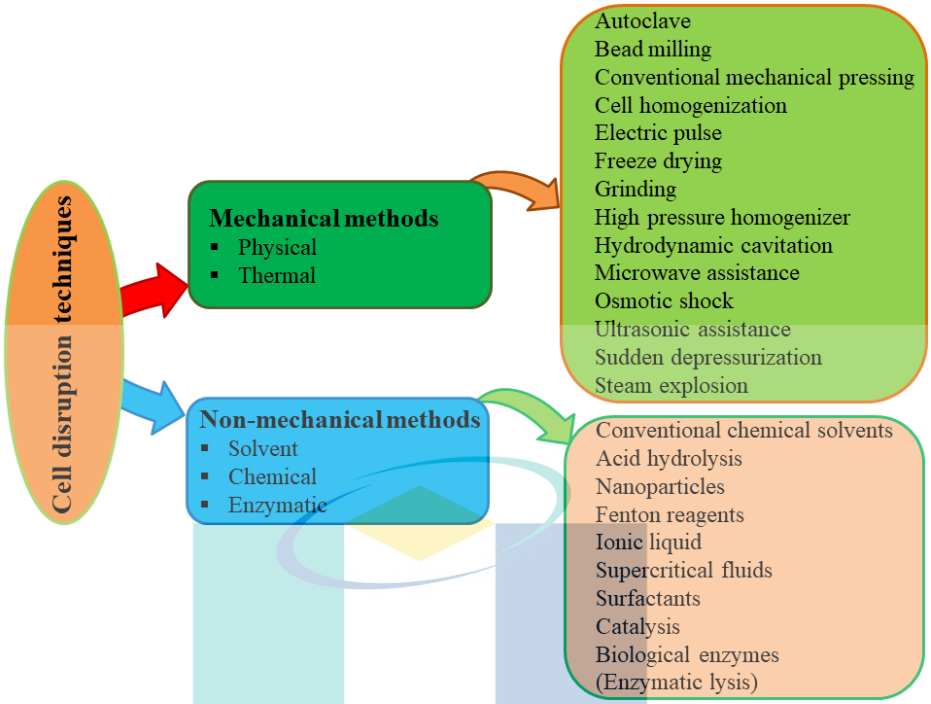

Figure 2.9 Different types of cell disruption techniques.

The most commonly used mechanical methods are bead milling; high pressure homogenizer; ultrasonication; microwave; autoclaving; electroporation (i.e., pulsed electric field); electrocoagulation; hydrodynamic cavitation; osmotic shocks; etc., and non-mechanical methods include enzymatic treatments, chemical treatments such as, Fenton's reagents; ionic liquids; supercritical fluids; surfactants; etc. (Patel et al., 2018). However, different pretreatment methods have their own mechanism to treat the algal cells, and the efficiency of a method mostly depends on the several operating parameters (Table 2.5). For instance, microwave shatters the cells using shock of high frequency waves (Cravotto et al., 2008), while ultrasonication cracks the cell wall and membrane due to a cavitation effect (Lee et al., 1998). On the other hand, brad beating causes direct mechanical damage to the cells based on high speed spinning with fine beads (Lee et al., 1998), whereas expeller press crushes and breaks the cells by high mechanical pressure (Enamala et al., 2018). In Fenton's method, the hydroxyl radicals (·OH) are generated via a reaction between H_2O_2 and Fe^{2+} ions ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {\cdot}OH$), and they can attack specific zones of the algal cell wall composed of organic compounds (Islam et al., 2018c). In electroporation, on the other hand, the higher treatment intensity of electrical field can induce irreversible permeabilization of the cell wall wading to its disruption by triggering fore formation (Onumaegbu et al., 2018; Patel et al., 2018).

Table 2.5 Summary of process parameters and mode of action for different cell wall disruption techniques.

Pretreatment methods	Mode of action	Control parameters	Energy consumption	References
Expeller/oil press	Mechanical compaction and shear forces	Pressure, configurations of various press (e.g., screw, expeller, piston, etc.)	High	(Onumaegbu et al., 2018; Patel et al., 2018)
High-pressure homogenization	Mechanical stress, the effect cavitation and shear forces	Number of passes used, operating temperature, pressure	High/medium	(Onumaegbu et al., 2018; Patel et al., 2018)
Ultrasonication	The effect of cavitation, the acoustic streaming and liquid shear stress	Frequency/Cycle number, power, time, temperature	Medium/low	(Onumaegbu et al., 2018; Patel et al., 2018)
Microwave	Temperature and molecular energy increase	Agitation, time, power, frequency	High/medium	(Onumaegbu et al., 2018; Patel et al., 2018)
Chemical treatments	Degradation mechanism, i.e., the protein, cellulose or/and pectin of microbial cell are degraded by different chemical reaction	Concentration of chemicals, such as NaOH and KOH	Low	(Onumaegbu et al., 2018; Patel et al., 2018)
Enzymatic treatments	Enzymes hydrolyze the chemical bonds by binding to specific molecules in the cell wall	Agitation, enzymatic type	Low	(Onumaegbu et al., 2018; Patel et al., 2018)
Autoclave	High thermal stress	Temperature, pressure, time	High/medium	(Onumaegbu et al., 2018; Patel et al., 2018)
Steam explosion	Sudden pressure drops, e. g., pressure wave and rapidly expanding steam can cause severe water hammer	Temperature, pressure, retention time, microbial species	High/medium	(Onumaegbu et al., 2018; Patel et al., 2018)
Pulsed electric field	Electroporation phenomena, including the electrochemical compression and electric field induced tension, cell membrane permeabilization and pore formation	Current intensity, voltage, electrode distance, conductivity, pulse duration	High/medium	(Onumaegbu et al., 2018; Patel et al., 2018)

Advantages and limitations of different pretreatment methods

The major concern during the selection of cell disruption techniques is the maintaining nutritive quality of products (i.e., lipids and other bioactive compounds) within the microbial cells. It is believed that some of the non-mechanical methods (i.e., chemical and enzymatic treatments) can modify the nutritive quality of the intracellular constituents (Lee et al., 2010). Moreover, these methods are often limited to the small scale and low process efficiency (Günerken et al., 2015; Islam et al., 2018c). Unfortunately, the practice of using chemicals and enzymes can lead to the greater complexity in minimizing environmental impacts. Consequently, these methods have found to be limited commercial applications to date (Islam et al., 2018c; Karim et al., 2018d), and are less favorable compared to some mechanical methods, such as bead milling (Günerken et al., 2015; Lee et al., 2012), ultrasonication (Günerken et al., 2015), and homogenizer (Lee et al., 2010). However, the mechanical methods also have varying degree of effects on microbial cells. For instance, bead milling could be used to completely break-up the cell wall leading to full disintegration of cells, but lipids are susceptible to degradation (Günerken et al., 2015; Lee et al., 2012). Although, the mechanical methods e.g., microwave, sonication, osmotic shocks, and high pressure homogenizer are preferred due to the high intracellular product release and better process efficiency; however, they are associated with high energy input, greater great generation, and longer time requirement (Günerken et al., 2015; Karim et al., 2018d). In addition, some these methods can also destroy the biomolecules of interest, such as denature of the proteins (Karim et al., 2018d). The main advantages and limitations of different cell disruption technique have been concised in Table 2.6.

Table 2.6 The advantages and limitations of different pretreatment techniques.

Pretreatment methods	Advantages	Limitations	References
Autoclave	Extraction is efficient and increased lipid yield, decreases the degradation of the desired product	High energy consumption, time consuming, not saleable	(Lee et al., 2010; Onumaegbu et al., 2018)
Bead milling	Can be scale up to a few m^3, no solvent uses, suitable for samples with high moisture content	May not extract majority of lipid, low efficiency with rigid cells, susceptible to lipid degradation, further process is required to remove undesirable products as well as beads	(Alhattab et al., 2019; Byreddy et al., 2016)
Fenton reagents	Fast, less energy consumption	Expensive chemicals, use of toxic chemicals and regents, chemical contamination during lipid extraction, high possibility to form inhibitors	(Concas et al., 2015; Onumaegbu et al., 2018)
Acid hydrolysis	Easy and simple method	Costly and toxic, biomass drying is required, high possibility to form inhibitors	(Sati et al., 2019)
Enzymatic	Mild operating temperature, low time and energy requirements, no harmful solvent uses, can be devoid of harsh physical conditions	Expensive, long reaction time, since it is depending on the cell wall characteristics; hence, less selective	(Alhattab et al., 2019; Sati et al., 2019)
Mechanical pressing	No solvent is required, simple and easy to use	Requires dry biomass, thus energy intensive, costly, and longer duration	(Bharte & Desai, 2018)
Microwave	Simple and rapid, can be scaled up, the energy requirement can range from 90-540 MJ kg^{-1}, reduced extraction time, environmentally friendly, reduced solvent usage, improved extraction yield	High temperatures may result in lipid oxidation, unpredictable efficiency, yet to be standardized, higher capital investment and operational cost	(Alhattab et al., 2019; Günerken et al., 2015; Onumaegbu et al., 2018; Patel et al., 2018)
Pulsed electric field	Higher yield, short time requirement, no chemical usage, no cell debris formation, relatively simple, eco-friendly	New method, has not been investigated with more algal species, further research needed, dependence on medium composition	(Bharte & Desai, 2018; Patel et al., 2018; Sati et al., 2019)
Ultrasonic pretreatment	Lower extraction time, less solvent requirements, higher penetration of solvent into cellular compartment, environmentally friendly	Energy intensive, difficulties in scale-up process, operational cost may be prohibitive	(Onumaegbu et al., 2018; Patel et al., 2018)
High pressure homogenization	Solvent free, easy and simple method, efficient extraction, short duration of contact time	Maintenance cost is high, less efficient with filamentous microbes	(Patel et al., 2018)

2.2.11 Electroporation based applications in energy and environmental microbiology

2.2.11.1 Electroporation

Electroporation (EP) is a well-known technique in the fields of medicine and biotechnology nowadays, however, many of its applications in the arena of environment and energy have only started to emerge; some of the most promising are reviewed here. Fundamentally, the exposure of the biological cells to a sufficeiently strong electric field leads to a significant and swift intensification of electric conductivity and permeability of the cell membranes, results in transient or permanent pores in the cell membranes, referred to as "electroporation" (Garcia et al., 2016; Masi et al., 2017; Miklavčič, 2012) or "electro-permeabilization". In this technique, the high electric pulses of direct current (DC) are applied to the living cells and tissues for a short duration of time to permeabilize the cell membrane for transfection or transformation (Garcia et al., 2016; Masi et al., 2017). These pulses are delivered to a pair of electrodes by a pulse generator (Garoma & Shackelford, 2014). Basically, a membrane potential is induced by an externally applied short and intense electric field (Kotnik et al., 2015). The pulse can be either a square-wave pulse, usually with a duration of less than a microsecond, or it can be an exponentially decaying capacitive discharge pulse with a duration in the millisecond ranges (Saunders et al., 1989).

EP is a microbiological technique that is today widely used to intensify the cell membrane penetrability by applying of a short-burst of high-voltage electric pulse to a sample placed between two electrodes (Yousuf et al., 2017a), allowing chemicals, drugs, or DNA to be inserted into the living cells (Garcia et al., 2016; Harak et al., 2017; Kandušer et al., 2017) or extract intra-cellular biomolecules from the cells (Kotnik et al., 2015; Sheng et al., 2011). It is massively exposed in many research that, small and/or large molecules can be introduced into cells or extracted from cells, proteins can be injected into the membrane, and cells can be fused by EP (Miklavčič et al., 2000). Since EP is applicable for all sorts of cells (such as microorganisms, plants, and animals) and for different purposes, without addition of viral or chemical compounds, it is considered as a universal method. As such, EP creates a diversified application in various areas of biotechnology, molecular biology, biochemistry, medicine, environment, and energy (Kandušer & Miklavčič, 2009; Miklavčič, 2012). In medicine, it is used for electro-

chemotherapy and gene therapy (Tao & Zhang, 2008). In biotechnology, it is used for transfection of bacteria, yeast, plant protoplast, and intact plant tissue (Teissie et al., 2002), and it is a prerequisite for cell electro-fusion (Ramos & Teissié, 2000). Currently, EP technology is enormously being used in food industry for liquid food sterilization and preservation (Kotnik et al., 2015; Yousuf et al., 2017a). It is also used for water sterilization and bacterial decontamination of hospital wastewater (Kotnik et al., 2015; Organization, 2014).

The unequal electric charges are accumulated on dipolar molecules, particularly diacylglycerols (DAG) in the cell membrane and peptidoglycan in the cell wall, while a biological cell passes through the high-strength (around 30 kV) and rapidly changing (around 2000 Hz) electric field (Salerno et al., 2009). The attractive forces of unequal electric charges can create strong pressure on the cell membrane, when a critical potential value of the electric field is exceeded (generally 1 V for bacteria), and high enough to form irreversible pores in the membrane (Sheng et al., 2011). This mechanism is called irreversible electroporation (IRE) (Weaver & Chizmadzhev, 1996) and the membrane also losses its fundamental properties of electrical resistance, membrane potential, and barrier function (Yousuf et al., 2017b). Moreover, EP has severe effects on the structure of biological tissues (Kotnik et al., 2015). Actually, a critical electric potential is tempted across the cell membrane by the application of a high intensity, an external electric field which leads to rapid electrical failure and local structural variations in the cell wall and cell membrane (Joshi & Schoenbach, 2000). Finally, the electric field results in a dramatic increase in mass permeability and, in some cases, mechanical rupture of the plant, animal or microbial tissues (Kotnik et al., 2015; Masi et al., 2017; Yousuf et al., 2017a).

2.2.11.2 Reversible and irreversible electroporation

The membrane of biological cells can be electrically pierced and lose its permeability temporarily or permanently while subjected to a strong electrical field (Weaver & Chizmadzhev, 1996). Depending on the process parameters (such as amplitude, duration, number and shape of the pulses, etc.), the electrical permeabilization of biological membranes can be either reversible or irreversible (Kandušer & Miklavčič, 2009). A transmembrane potential is induced while an external strong electrical field is applied to the cell, and the induced potential difference across the membrane is

proportional to the external electrical field intensity (Sale & Hamilton, 1967). The induced trans-membrane voltage imposed by external electric field is required to reach a critical value to generate the formation of transient aqueous pores in the cell membrane (Kinosita & Tsong, 1979). The membrane potential is required to maintain below the critical value for reversibility of electroporation. In such conditions, the cell membrane can able to recover after electric pulse application, the pores in the cell membrane can reseal and the membrane will return to the initial and normal state, thus the cell survives (Jordan et al., 2013). On the other hand, when the critical value of the membrane potential is exceeded, irreversible electroporation takes place, resulting in the disintegration of the cell membrane and loss of cell viability (Gusbeth et al., 2009b) (Figure 2.10).

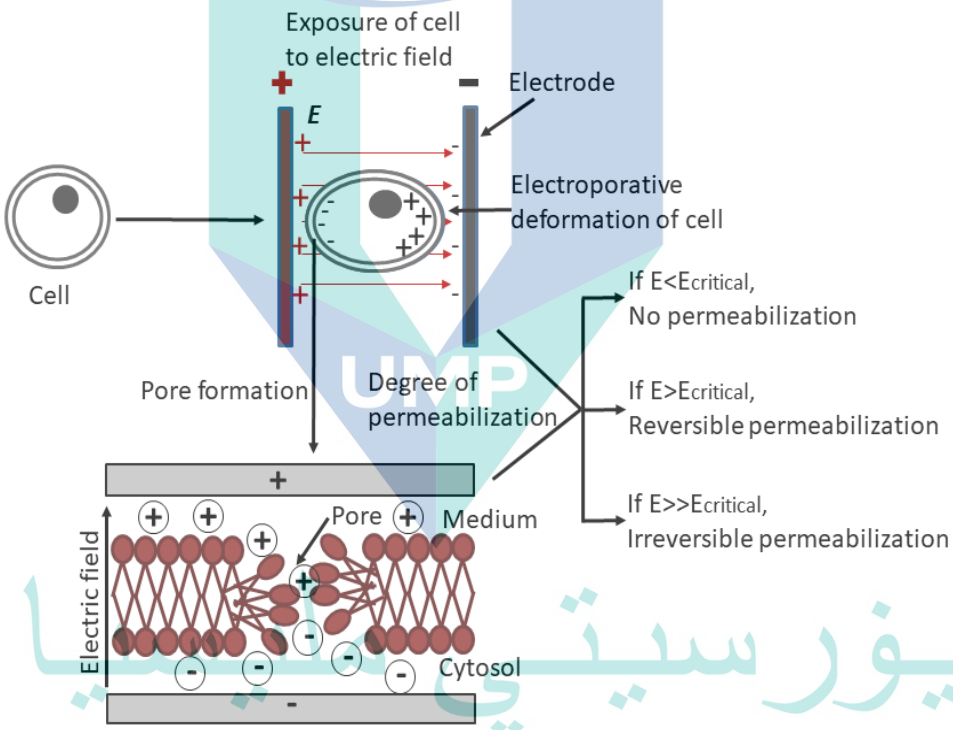

Figure 2.10 The mechanism of electroporation through the cell membrane permeabilization. When a high-strength and rapidly changing electric field is exposed to a biological cell, the positive charges outside the cell and the negative charges inside the cell are attracted to the electrodes leading to electro-compression and subsequent electro-permeabilization of the plasma membrane leads the cells to dead .

Source: Modified based on Raschke (2010)

Temporary and limited pathways for molecular transport through nanopores are formed in reversible electroporation (RE), but after the termination of the electric pulse, they progressively reseal, the transportation of molecules stops, and most cells retain their viability. On the contrary, the permanent and lethal nano-pores in the cell membrane are

created by inducing short but strong electrical fields to disrupt the cellular homeostasis in the IRE. Subsequently, a certain degree of damage to the cell membranes is occurred by electroporation. Then the leakage of intracellular contents is too severe, or the resealing of the cellular membrane is too sluggish to preserve cell viability, leaving healthy cells to gradually disintegrate and irreversibly damaged. Consequently, the cells release their contents, but released contents are not thermally yet damaged. Lastly, in the case of irreversible electroporation with thermal damage, the electric current causes an adequate increase in temperature to cause thermal damage of the released molecules (i.e. protein denaturation above 50 ºC and DNA melting above 70 ºC) (Kotnik et al., 2015) (Figure 2.11).

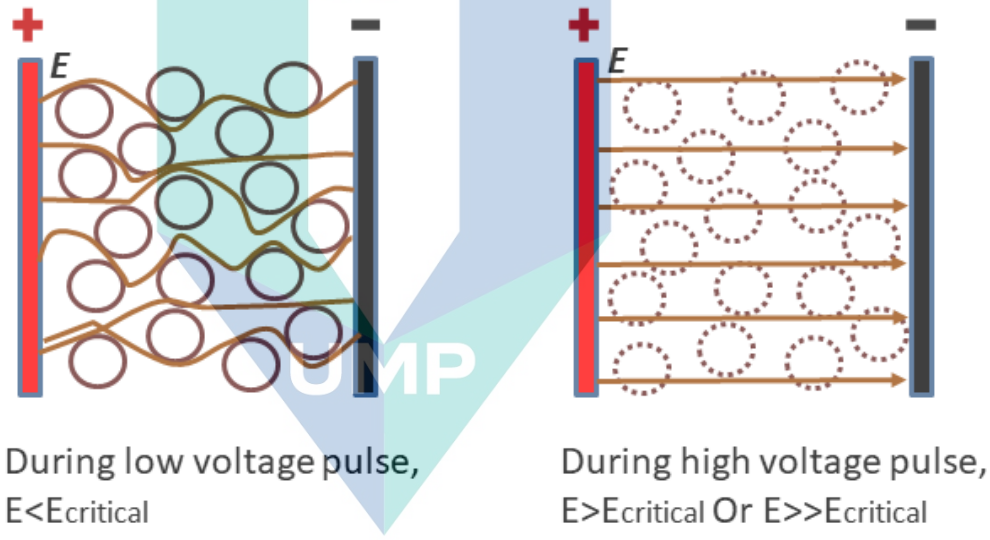

Figure 2.11 The characteristics of the electric current pathway through cells in tissue without (left) and with (right) electroporation occurrence. The increases in electrical conductivity is directly correlated with the intensity of electric field exposure. As soon as the electrical field is induced to the cells of the tissue, the electroporated cells in this region no longer serve as dielectrics in the tissue, permitting improved electrolyte mobility within the environment and thus increased conductivity.

Source: Modified from Yano et al. (2017)

However, the reversible breakdown of the cell membrane has wide applications in biotechnology (transfection of bacteria, yeast, plant protoplast and intact plant tissue etc.) and medicine (electro-chemotherapy and gene therapy etc.) (Hofmann & Evans, 1986), while the applications of irreversible breakdown are mainly in food industry (especially liquid food sterilization), public health, pharmaceutical research and water purification (Qin et al., 1994) (Figure 2.12).

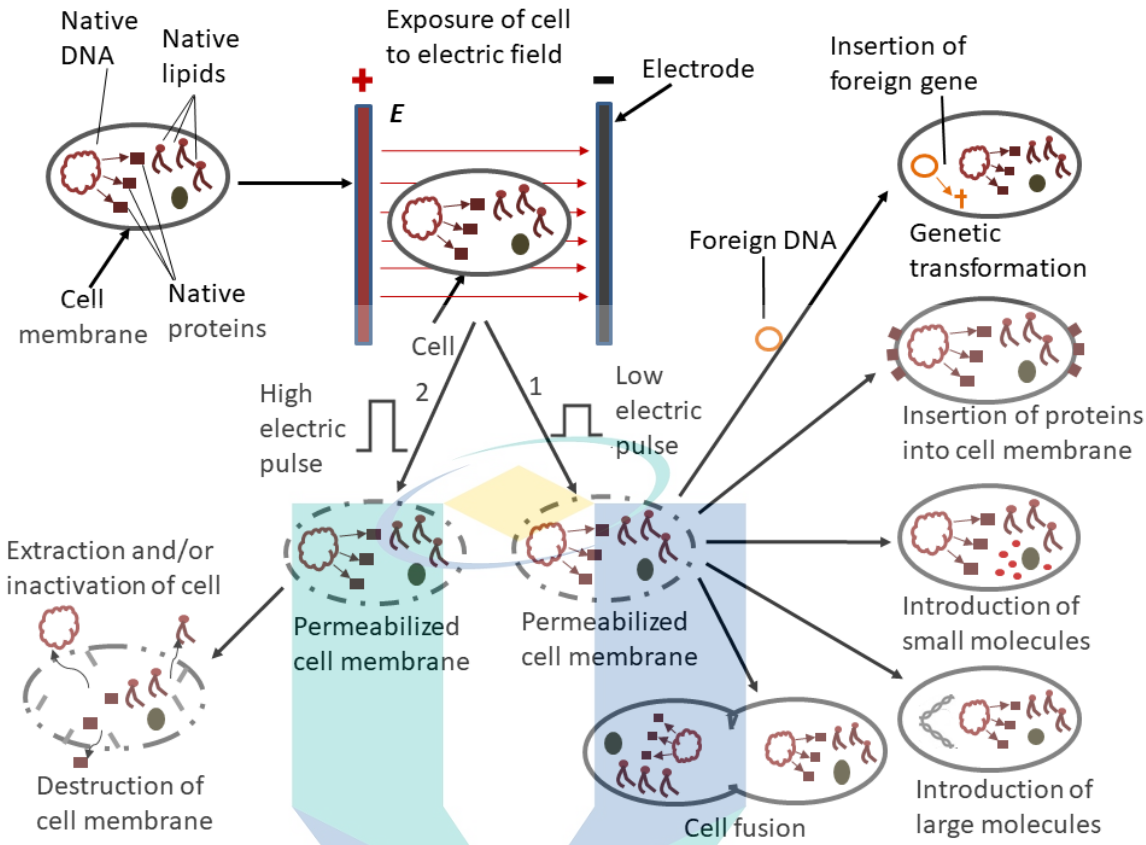

Figure 2.12 Biotechnological applications of EP for a single cell, Route-1: Reversible EP, the external electric field can permeabilize the cell membrane when it reaches to the threshold values of the cell membrane to insert the protein, large and small molecules inside the cell or can occur cell fusion, if two single cells are close to each other. Route-2: Irreversible EP, when applied intense electric field exceeds the certain critical value, destruction of the cell membrane can occur resulting cell death.

Source: Adopted from Yousuf et al. (2017b).

2.2.11.3 Responses of macro- and micro-molecules to electroporation

The field of EP applications prolonged significantly (Weaver et al., 2012) and it was revealed that if the sufficiently intense electric field applied to a living cell, the cell loses its homeostasis and eventually dies, which is called irreversible electroporation (Yu et al., 2012). The method gained a strong ground as a tool for microbial inactivation (Kandušer et al., 2017; Sale & Hamilton, 1967) and the effect of pulsed electric fields on microbial viability has been extensively studied on various Gram-positive bacteria (Garcia et al., 2016; Yeo & Liong, 2013), Gram-negative bacteria (Garcia et al., 2016; Haberl et al., 2013; Žgalin et al., 2012), yeasts (Karim et al., 2018c), protozoa parasites (Haas & Aturaliye, 1999) and even spores (Marquez et al., 1997). Since microbial inactivation by EP in controlled laboratory conditions exhibited potential (Figure 2.13),

the idea becomes emerged in removing pathogenic microbial agents from several water sources (Gusbeth et al., 2009b; Rieder et al., 2008) and from liquid food, without abolishing vitamins or disturbing the food's texture, flavor or color (Saulis, 2010; Yeo & Liong, 2013). EP has also been used to take out molecules from cells (Grimi et al., 2014), for instance, plasmid DNA from bacterial cells (Band et al., 2016; Haberl et al., 2013), proteins from various microorganisms (Coustets et al., 2013; Zhan et al., 2010), sugar from sugar beet cells (Loginova et al., 2011; Sack et al., 2010) and oil biodiesel from oil-producing microalgae (Coustets et al., 2013; Goettel et al., 2013; Zbinden et al., 2013).

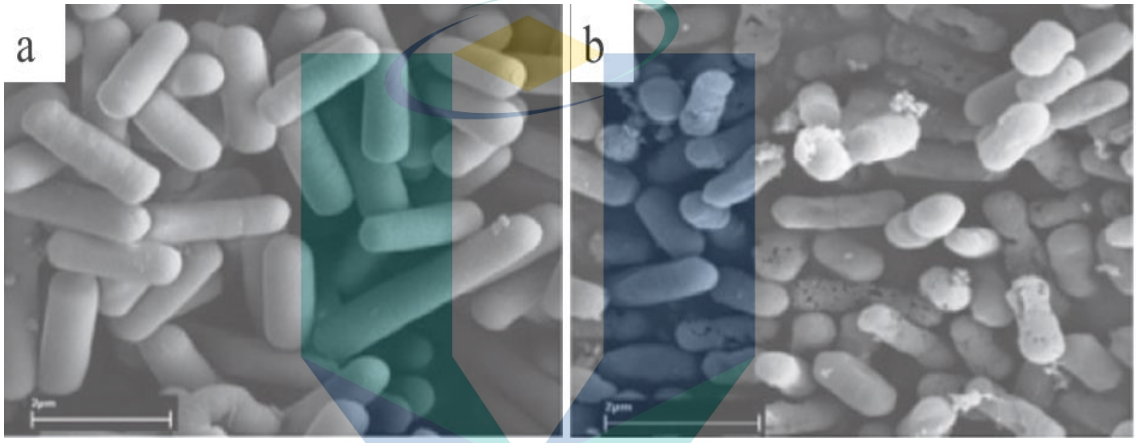

Figure 2.13 Scanning electron micrographs of *Lactobacillus casei* bacteria a) before and b) after exposure to an electric field pulse of 7.5 kV/cm amplitude and 4 ms duration (scale bar corresponds to 2 mm).

Source: Yeo and Liong (2013)

EP can create conditions for the transportations of small or large molecules through the intra-cellular membrane and probably, that is the most important functional application of it (Granot & Rubinsky, 2010). As an example, this phenomenon is being applied to intensify the insertion of nucleic acid molecules in genetic modifications (Jordan et al., 2013), to augment drug transportation in cancer treatment termed as electro-chemotherapy (Mir & Orlowski, 1999) and many other applications. In the last decade, an increased attention to extractive recovery with EP has been established for different food plants and biomass feed-stocks (Brianceau et al., 2015; Vidal, 2014) (Figure 2.14).

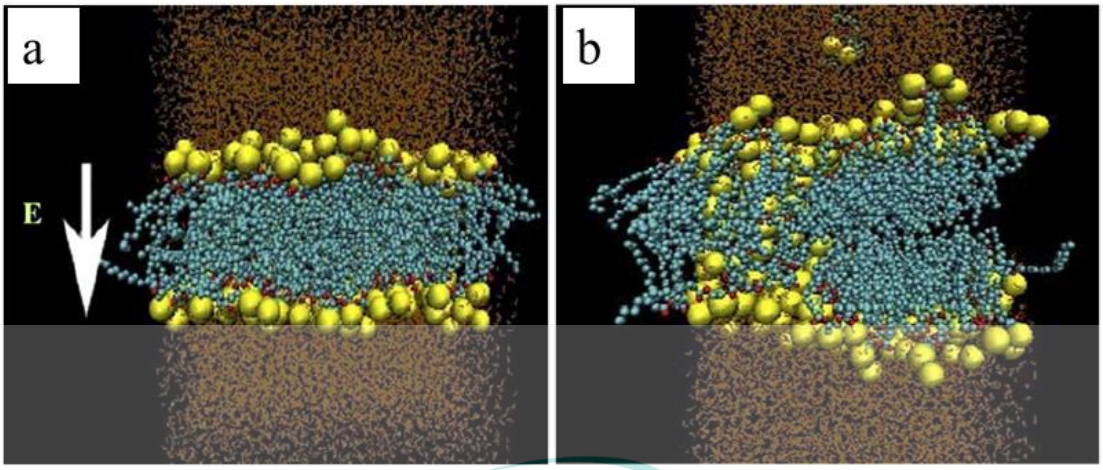

Figure 2.14 (a) The initial configuration of a dipalmitoyl-phosphatidyl-choline (DPPC) lipid bilayer used for the molecular dynamics' simulations. Water is shown in brown, lipid headgroups in yellow and lipid chains in cyan. (b) A 2.05 ns simulation snapshot of the DPPC lipid bilayer patch with the voltage pulse applied. One lipid at the anode side (top) is seen to break loose.

Source: Pliquett et al. (2007).

2.2.11.4 Influential parameters of irreversible electroporation

Although EP is used in an extensive range of diverse cell types, biological characteristics of the treated cell, e.g., membrane fluidity, integrity of cytoskeleton, and presence of cell wall in bacteria, yeast, and plant cells, affect its effectiveness. Moreover, the value of induced trans-membrane voltage depends on the cell shape, size, and the position of the cell with respect to the direction of the applied electric field (Valič et al., 2004). It is important to consider the specific features of different cells when optimizing electroporation parameters. However, the efficiency of the micro-organism inactivation by EP depends on a variety of factors such as electric treatment (amplitude, duration and shape of the pulses, their number), biological (size, concentration, growth phase, and strain of microorganisms), and physical-chemical (temperature, pH, ionic composition, conductivity, osmotic pressure and ionic strength of the medium) etc. (Gómez et al., 2005). The parameters of electric pulses were extensively investigated. Several studies have investigated the inactivation of bacteria predominantly in relation to microbial inactivation in liquid food (Wu et al., 2005) and wastewater (Rieder et al., 2008). Outcomes of those studies exhibited significant inactivation but with variable results depending on the microorganisms, the medium in which they were treated and the different electrical parameters that were used. Electrical field strengths ranged from 10 to

40 kV/cm, with pulse duration from 1 µs to 100 ms was mostly studied (Mosqueda-Melgar et al., 2007). If the electrical field strength exceeds the critical value (E_c) for a given length of time (t_c), permanent holes are formed and considered as irreversible electroporation. The resulting inactivation of microorganisms is considered to be related to both the electrical field strength and the total time of treatment (Hülsheger et al., 1983).

2.2.11.5 Application of electroporation in wastewater treatment

EP assisted disinfection is a promising technology for the non-thermal disinfection of water. The EP has been already established for inactivation of micro-organisms in the 1960s and verified to be capable of disinfecting drinking water and liquid food (Drees et al., 2003; Saldaña et al., 2014). The bacterial decontamination of hospital wastewater by irreversible electroporation is emerging approach in wastewater treatment. EP or Pulsed electric field (PEF) could be able to limit the spread of harmful bacteria into the environment by eliminating antibiotic-resistant strains, which is of universal concern at the present time (Organization, 2014). The applicability of an alternative wastewater disinfection concept based on the PEF treatment is tested with molecular biology techniques using clinical wastewaters by Rieder et al. (2008). The results indicated that the PEF treatment is an appropriate alternative disinfection concept for the treatment of clinical wastewaters and surpass the disadvantages of other disinfection methods. The bacterial population of wastewater with a temperature below 70 ºC can be reduced by four orders of magnitude at an energy input of 150 kJ/L (Rieder et al., 2008). Moreover, the required treatment energy for efficient disinfection could be reduced to 40 kJ/L by a combination of pre-heating to 60 ºC and subsequent electroporation has proved synergistic (Gusbeth et al., 2009a). The use of the short duration electrical pulses from the magnetic pulse compressor for inactivation of spores, bacteria (*E. coli*) and viruses in drinking water was investigated by Narsetti et al. (2006). The inactivation of Gram-positive strains was also found to be effective with this combination, which is tougher to inactivate by EP alone. Generally, bacteria readily develop tolerance while disinfected with ultraviolet light. But it was revealed that decontamination with EP does not lead to bacteria developing tolerance or resistance to the treatment for at least 30 generations (Gusbeth et al., 2009b). The effects of EP to inactivate the *E. coli* in distilled water were investigated by Žgalin et al. (2012), where 2-log reductions in bacterial counts were achieved at a field strength of 30 kV/cm with eight pulses and a 4.5-log reduction was

observed at the same field strength using 48 pulses. Kotnik et al. (2015) demonstrated that the EP as a disinfection technology which is also efficient under high mass flow conditions by up scaling the system to a pilot scale flow of 400 L/h (Figure 2.15).

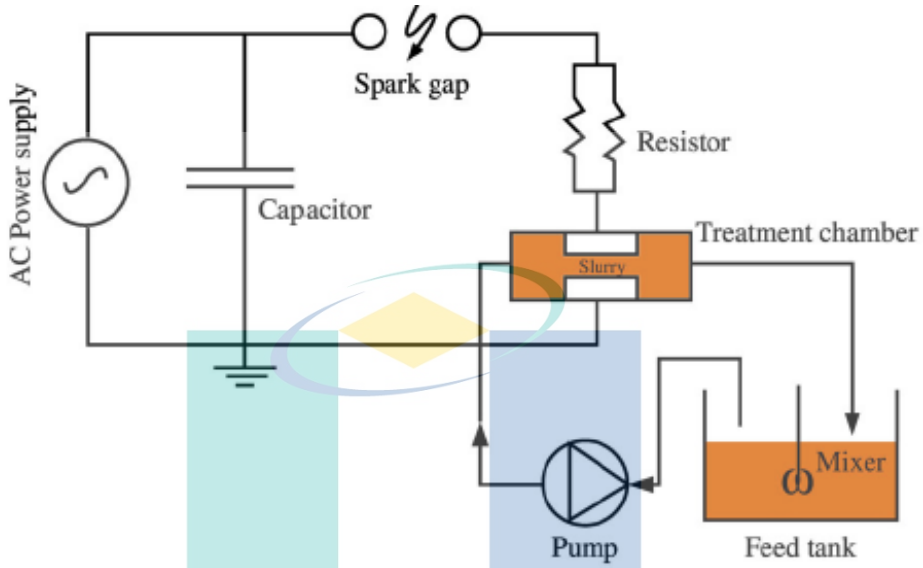

Figure 2.15 Schematic diagram of process flow of pulsed electric field (PEF) pre-treatment set-up. The substrate was driven to PEF treatment chamber from the feed tank and directed back to the tank. The homogenizer and the pump were remained continuously running to maintain the samples homogenized.

Source: Safavi and Unnthorsson (2017)

Generally, the cell wall is the main target for microbial inactivation process since it maintains the integrity and morphology of bacteria. Pillet et al. (2016) revealed that the cell wall and coat architecture are directly involved in the electro-eradication of bacteria. They stated that irreversible cell membrane electro-permeabilization causes cell-wall organization of living *Bacillus pumilus*, thus the death of the bacteria which is one of the promising approaches of PEF to inactivate the bacteria. They demonstrated that the exposure to PEF led to structural disorganization correlated with morphological and mechanical alterations of the cell wall for vegetative bacteria. On the contrary, PEF exposure led to the partial destruction of coat protein nanostructures, associated with internal alterations of cortex and core for spores. However, the inactivation of pure cultures by applying different electric fields has been demonstrated in several publications (Hülsheger et al., 1981; Martin et al., 1997; Pillet et al., 2016; Qin et al., 1994; Spilimbergo et al., 2003). In a study, the *Escherichia coli* populations were quickly reduced with the application of EP at initial pulses (12·34 kV/cm and 2·7 pulses) and then

a minor effect was observed on the microbial cell reduction with the subsequent pulses (30 kV/cm and 30 pulses) (Martin et al., 1997; Qin et al., 1994). A similar trend was recorded in the another study, where 48% of the anaerobes were rapidly inactivated within the first 30 seconds (Karim et al., 2018a). Spilimbergo et al. (2003) stated that the viability of bacteria (*E. coli*, *Staphylococcus aureus*) might be lessened with the increasing of the number of pulses and electric field intensity. In another study, the inactivation of pathogens in liquid was reported for water disinfection by applying a high electric field strength (>107 V/m) with a low-voltage (1 V) electroporation disinfection cell and investigate the critical mechanisms of cell transport to allow high inactivation performance (Huo et al., 2018). Therefore, EP could be a promising technique for wastewater treatment.

2.2.11.6 Application of electroporation in biogas production

Lignocellulose is the most abundant and readily available biomaterial in nature, but use is still limited due to their complex structure and high processing cost. The complex structures of lignocellulosic biomass are difficult to break down and thus require longer retention times for the nutrients to become biologically available (Yousuf, 2012; Yousuf et al., 2017d). It is possible to increase the digestibility of the substrate by pre-treating the material before digestion (Yousuf et al., 2017a). Chemical methods are often limited to small scale and usually result in less intracellular product release and low process efficiency. Additionally, the practice of using chemicals and enzymes can lead to greater complexity in minimizing environmental impact (Byreddy et al., 2015; Günerken et al., 2015; Yousuf et al., 2017d). On the other hand, physical methods associated with high energy input while the rate of hydrolysis is very low for biological methods. Consequently, those have found limited commercial application to date. Though, physico-chemical methods are popular due to high product yield, they are associated with excessive cost, partial hemicellulose degradation, alters lignin structure, etc. (Günerken et al., 2015; Liu et al., 2016). Therefore, electroporation could be an efficient pretreatment to overcome those limitations. Lindmark et al. (2014) explored that, it is possible to increase the digestibility of the substrate by pre-treating the material before digestion by a pre-treatment. The high electric fields of direct current, known as EP, was applied to pre-treat the ley crop silage and to investigate the potential of EP technology for enhancing biogas production from ley crop silage (Figure 2.16). The results showed that

it was possible to intensify the biogas yield with 16 % by exposing the substrates to 65 pulses at a field strength of 96 kV/cm corresponding to a total energy input of 259 Wh/kg volatile solid (VS). However, the energy balance of the EP treatment suggests that the yield, in the form of methane, can be up to double the electrical energy input of the process (Lindmark et al., 2014). EP as a novel pretreatment method using electric field was employed by Đurđica et al. (2019) for pre-treating the lignocellulose substrates to enhance biogas production through anaerobic co-digestion with dairy cow manure.

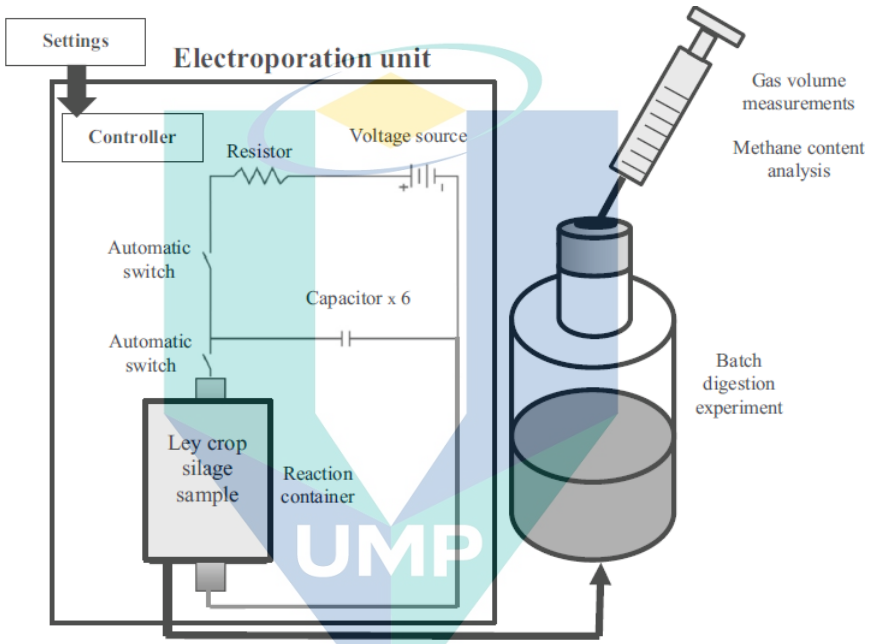

Figure 2.16 Experimental setup of the electroporation unit and the batch digestion experiment was performed to assess the effects of electroporation on ley crop silage. Source: Adopted from Lindmark et al. (2014)

EP has previously been studied for the treatment of sewage sludge (Choi et al., 2006; Rittmann et al., 2008), pig manure (Salerno et al., 2009), and source-sorted municipal organic solid waste (Uldal et al., 2009) before digestion in a biogas plant. Choi et al. (2006) studied, the soluble chemical oxygen demand (SCOD)/total chemical oxygen demand (TCOD) ratio and exocellular polymers (ECP) content of waste activated sludge increased 4.5 times and 6.5 times, respectively by pulse-power pretreatment (Figure 2.17). Batch-anaerobic digestion of pulse-power pre-treated waste activated sludge was able to enhance biogas production 2.5-fold compared to untreated material (Choi et al., 2006), and pre-treatment of municipal solid waste augmented biogas production by up to 14 % (Uldal et al., 2009).

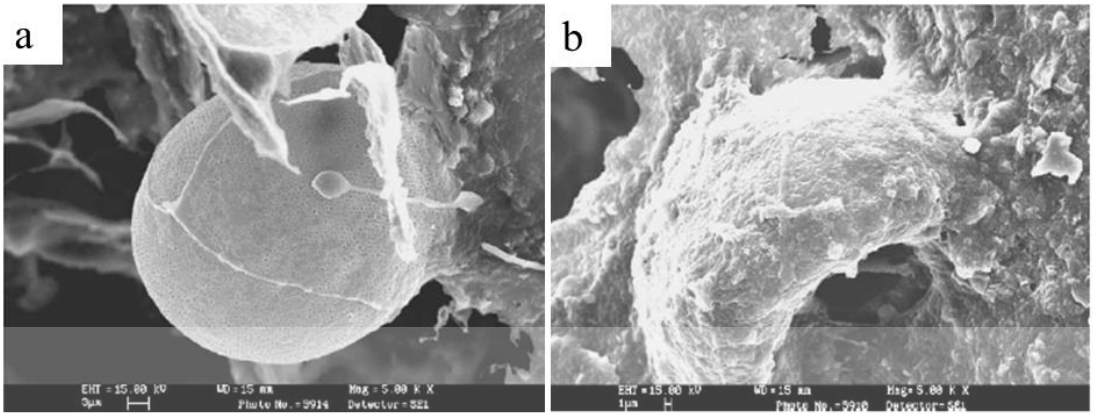

Figure 2.17 SEM images of sludge cells: (a) raw activated sludge cell; (b) pulse-power treated sludge cell. SEM images clearly showed that pulse-power pretreatment of WAS resulted in the destruction of sludge cells.

Source: Adopted from Choi et al. (2006)

2.2.11.7 Electroporation in bioethanol production

EP can be a potential technique as a pretreatment method for bioethanol production from lignocellulosic biomass as it is a technique to apply a high voltage for a very short duration to disrupt the cell structure and increase the porosity of biomass, so it might be produced fermentable sugar from lignocellulosic biomass (Yousuf et al., 2017a). As a result, it will enhance the bioethanol production through accelerating the fermentation process. High pulsed electrical field has been applied as a pre-treatment method by Almohammed et al. (2016) for sugar beet tails as a sustainable feedstock for bioethanol production (Figure 2.18). The yield of solutes was increased from 16.8% to 79.85% and the dryness of pressed cake was increased from 15% to 24% in comparison with the untreated tails at an intensity, E = 450 V/cm and duration, tPEF = 10 ms corresponding to an energy input Q = 1.91Wh/kg. It was demonstrated that the higher content of fermentable sugars in PEF expressed juice leads to a higher ethanol content in distillate (6.1% vs. 2.95% v/v) and a higher CO_2 weight loss (57.2 vs. 28.3 g/L) than that obtained from the raw juice of untreated tissue (Almohammed et al., 2016).

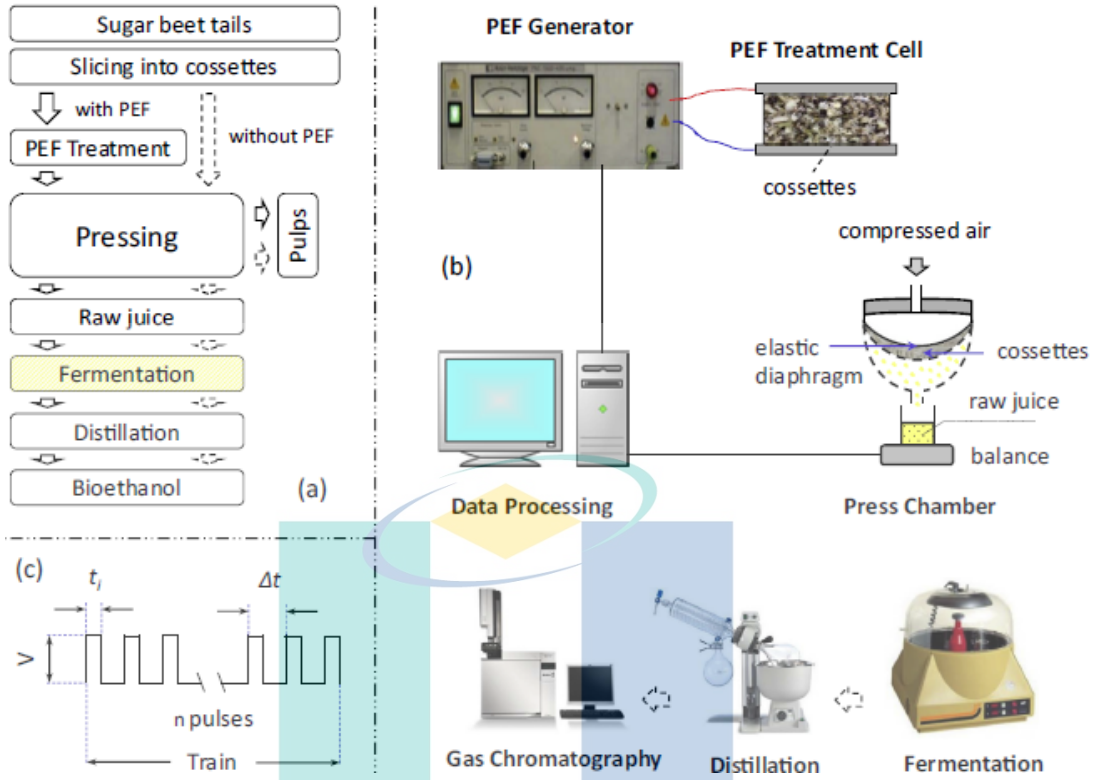

Figure 2.18 Schematic diagram for valorization process of sugar beet tails (a), experimental setup (b), and PEF pulsing procedure (c)

Source: Adopted from Almohammed et al. (2016)

2.2.11.8 Electroporation in biohydrogen production

Cellulose and lignocelluloses including vegetables and fruits that contain many kinds of fermentable sugars are the most abundant biopolymers from plants and could be considered as a valuable feedstock for biohydrogen production (Lalaurette et al., 2009). Biohydrogen production from food waste and food processing waste containing large amounts of cellulose like jackfruits peels (Vijayaraghavan et al., 2006), apple waste (Hwang et al., 2011), citrus waste (Karim et al., 2018a), and vegetable kitchen waste (Lee et al., 2008) results in diverse and interesting biohydrogen yields. However, it is proven that degradation of cellulose is very difficult by biological treatments due to its crystalline and rigid structure (Lee et al., 2008). In a recent study, Jeong et al. (2015) revealed the practicability of EP (20-100 V for 30 min) as a pretreatment technique to increase biohydrogen production from marine brown algae (e.g., Laminaria japonica) biomass feedstock. They demonstrated that the hydrogen yield was enhanced by 72.6% at 58.5 V for 30 min which indicating the potential of the EP as alternative technique for feedstock preparation. The highest hydrogen yield was obtained at a voltage of 58.5 V because of

the enhanced biodigestibilty of substrate. This has been ascribed to the fact that the structural bonding of LCB would be broken down due to the application of an external electric field (Figure 2.19), which in turn can promote the enzymatic accessibility to cellulose and hemicellulose for hydrolysis by fermentative bacteria (Almohammed et al., 2016; Yousuf et al., 2017a).

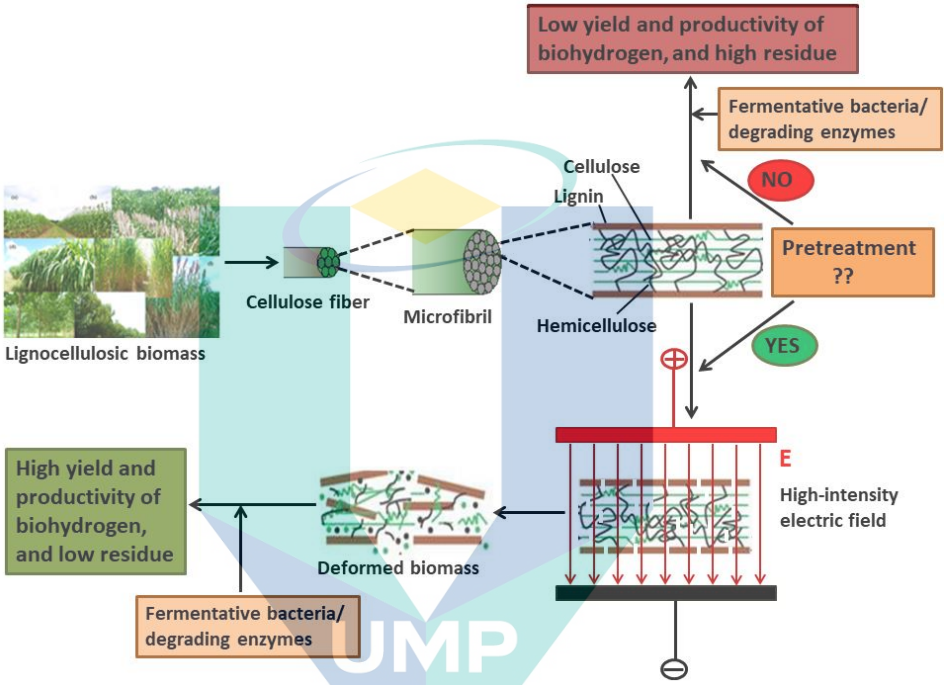

Figure 2.19 Conceptual effect of EP on lignocellulosic biomass.

On the other hand, mixed culture microorganisms particularly anaerobic sludge (AS), are considered effective inoculum compared to pure cultures in fermentation system to produce biohydrogen. However, AS contains various types of microbes, including hydrogen-producing and hydrogen-consuming bacteria (i.e. homo-acetogens and methanogens) (Rajesh et al., 2015). Therefore, hydrogen-consuming bacteria, especially methanogens (since methanogens are dominant in AS) must be suppressed in order to enhance the performance of hydrogen production through anaerobic fermentation (Karim et al., 2018a). Recently, EP has been used to inactivate the methanogens from AS in order to enrich the hydrogen producers (Figure 2.20), and consequently to enhance the hydrogen production (Karim et al., 2018a). The result of this study showed a drastic increase (315%) in hydrogen yield using 1 min EP (TI=60 kWh/m^3) treated inoculum (AS). Therefore, EP could be an effective pretreatment in biohydrogen production not only for substrate, but also for mixed culture inoculum.

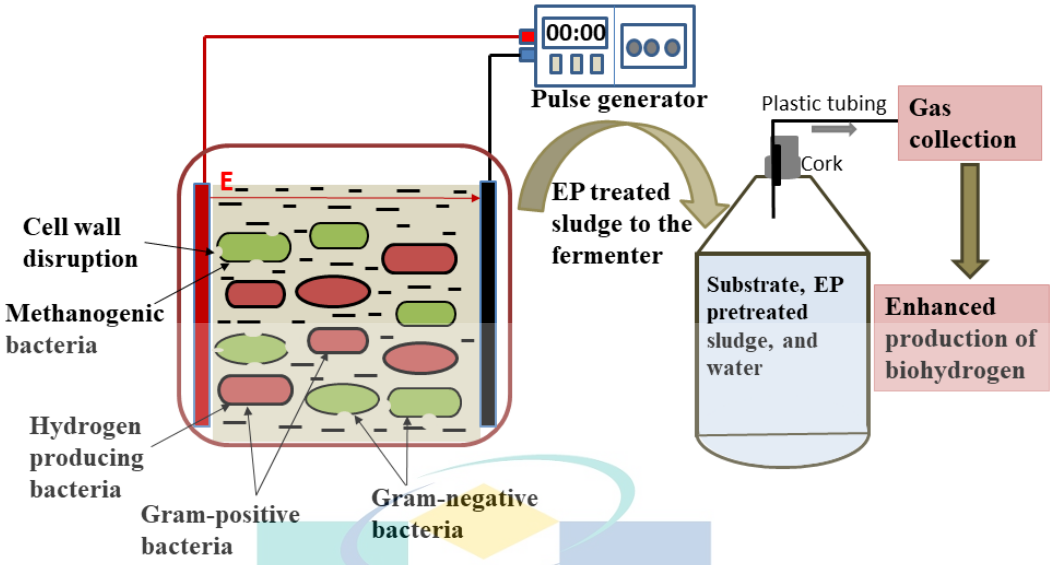

Figure 2.20 Schematic diagram of EP system for pretreatment of mixed culture sludge to enhance biohydrogen production.

Source: Karim et al. (2018b).

2.2.11.9 Electroporation in biodiesel production

Biodiesel production from microbial oils or SCOs has become very widespread in recent days. Microalgae and yeast are promising feedstock for the production of biodiesel due to their high lipid content and possess great advantages such as higher growth rate and productivity, grow in various environments (fresh, brackish or salt water), do not compete for land, and have high oil productivity (20%–50% by dry weight basis) compared to conventional crops (Yousuf & Pirozzi, 2009). However, the challenging part of the biodiesel synthesis from SCO is the extraction of microbial oils from intracellular compartments (Yousuf et al., 2010). EP could be an effective technique in the stage of oil/lipid extraction process. Transesterification can be completed *in situ* by applying EP, also called direct transesterification, where the reaction is carried out in a single step (Figure 2.21). It is assumed that EP technology could enhance the transesterification process triggering pores in the microbial cell wall while exposed to electrical fields. During the treatment, the cells would be damaged and release the intercellular nutrients to surrounding media that can lead to an increase in the biodiesel production rate (Yousuf et al., 2017a).

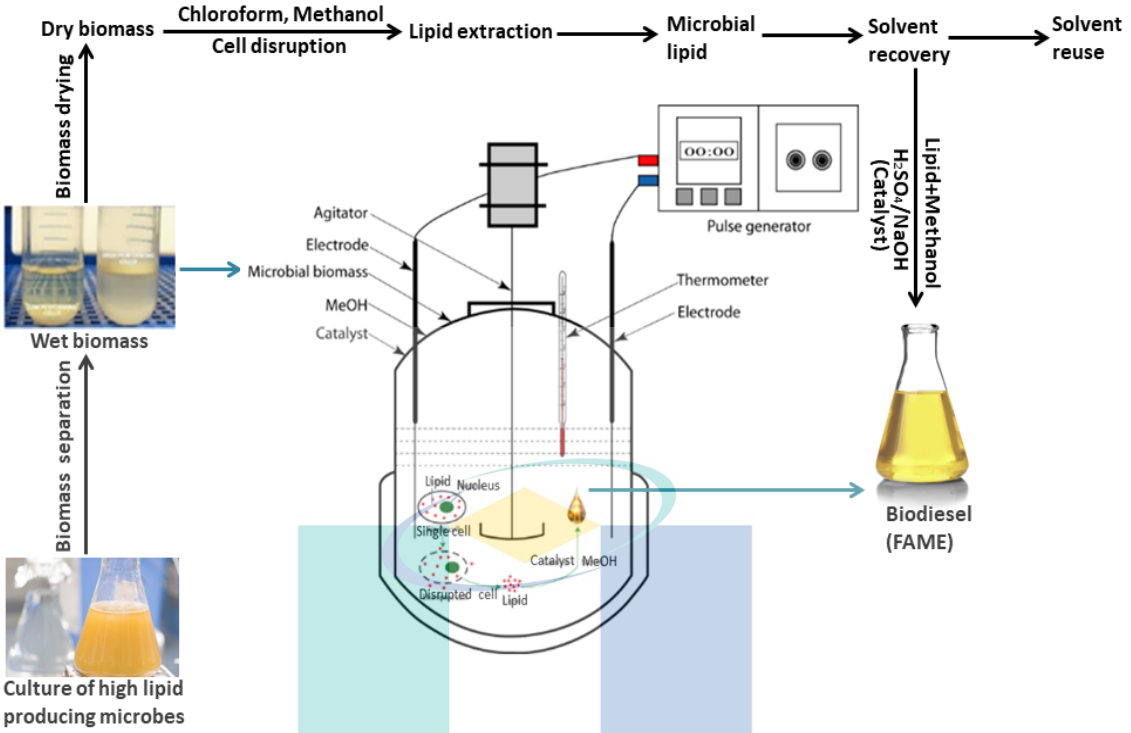

Figure 2.21 Process difference between traditional and direct transesterification with EP in microbial lipid synthesis for biodiesel production.

The use of wet biomass opens a promising processing route for an energetic use of microalgae, because the energy consumed conventionally for drying of the biomass is considerably higher than the energy required for PEF treatment (Goettel et al., 2013). PEF technology was employed by Zbinden et al. (2013) as a process strengthening strategy to boost up lipid extraction from wet biomass of *Ankistrodesmus falcatus* using the green solvent, ethyl acetate. It was reported that 90% of the cells could be lysed by utilizing PEF technology and a substantial enrichment in the rate of lipid recovery using ethyl acetate. They also stated that the increase in lipid recovery was due to the presence of the electric field and not due to temperature effects (Zbinden et al., 2013). In another study, the PEF treatment with 23–43 kV/cm electric field strength and a duration of 1 µs was applied for the cell disintegration of the freshwater microalgae *Auxenochlorella protothecoides* (Goettel et al., 2013). The continuous flow PEF treatment for *E. coli* inactivation and lipid extraction from microalgae *Chlorella vulgaris* was studied by Flisar et al. (2014). In this study, lipids were extracted via concentration of biomass, drying and cell rupture using pressure or an organic solvent in control experiments. In contrast, EP bypasses all stages, since cells were directly ruptured in the broth and the oil that floated on the broth was skimmed off. The preliminary experiments showed a 50% oil yield using the EP flow system in comparison to extraction with organic solvent (Flisar et al., 2014).

PEF technique was studied for cell disruption prior to intracellular lipid extraction from *Synechocystis* PCC 6803 as feedstock of nonpetroleum-based diesel fuel by Sheng et al. (2011). Severe cell disruption was evident after PEF treatment, especially with treatment intensity (TI) > 35 kWh/m3. Seven cell-disruption methods – autoclaving, bead beating, freeze drying, french press, microwave, PEF, and ultrasound – were tested prior to lipid extraction to make intracellular lipids more accessible by organic solvents in order to extract intracellular lipids from cyanobacteria *Synechocystis* PCC 6803 for biofuel production Sheng et al. (2012). Severe cell disruption was evident after PEF treatment, especially with TI > 35 kWh/m^3. It was found that the microwave, PEF, and ultrasound with temperature control had noteworthy enhancement of lipid extraction (9–13% increases), however, microwave and PEF (with temperature control) might be best suited for large-scale cell disruption among all methods reported in this study in order to minimize the cost of cell-disruption and lipid-extraction steps.

2.3 Single cell oil (or microbial lipid) and its application for biodiesel production

2.3.1 Overview of microbial lipid

Biodiesels are fatty acid methyl esters derived from renewable lipid sources, these fuels are good alternatives for fossil fuel because of being renewable, biodegradable, nontoxic, inherent lubricity, superior flash point and negligible sulfur content (Chatzifragkou et al., 2011; Karatay & Dönmez, 2010; Kosa & Ragauskas, 2011). Biodiesel can be produced by splitting oils and reacting with them by a simple alcohol (Milano et al., 2016). If vegetable oil or animal fat is used for biodiesel production the cost of substrate will be 70–85% of the total cost so biodiesel production from these sources is not suitable. The mentioned sources are used by humans so there is no possibility for large-scale production (Madani et al., 2017; Milano et al., 2016).

There are oleaginous microorganisms such as yeasts, fungi and micro-algae which can accumulate high amounts of reserved lipids under appropriate cultivation condition (Figure 2.22), so their potential as lipid producing sources has attracted high attention and can be used as feedstock for biodiesel production (Kumar et al., 2020; Muniraj et al., 2013). Substituting of microbial lipid instead of plant's oil for biodiesel production is a developing idea (Milano et al., 2016). The oil obtained from yeasts and

fungi has high similarity with plant's oil and make it appropriate for biodiesel feedstock so application of microbial oil reduces the cost of biodiesel production (Madani et al., 2017; Sriwongchai et al., 2013).

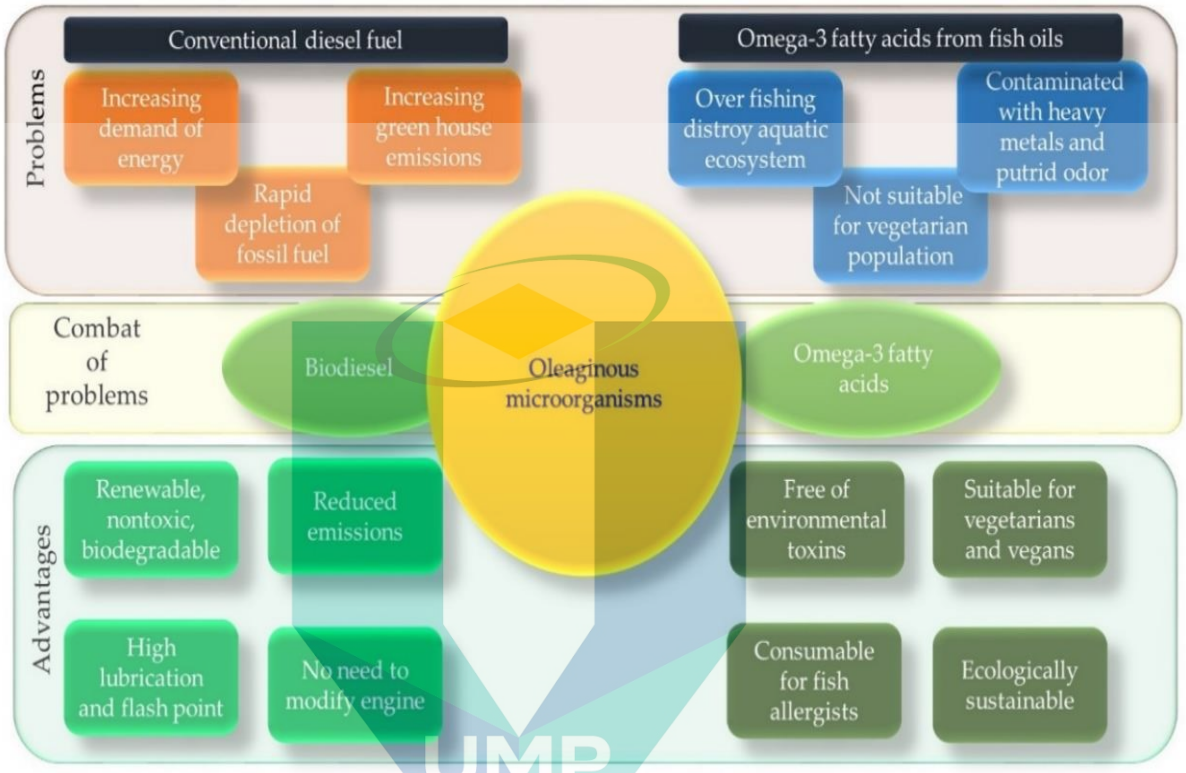

Figure 2.22 Based on the fatty acid profiles, oleaginous microorganisms can be used for biodiesel production or nutraceuticals.

Source: Patel et al. (2020)

Unicellular microbes like yeasts and bacteria have high growth rate and can accumulate lipid in separate lipid bodies (Kumar et al., 2020). The advantages of microbial lipid are short life cycle of microorganisms, less labor required, less affection by season and climate and easier to scale up (Patel et al., 2020). Oleaginous yeasts accumulate triacylglycerol rich in unsaturated fatty acids. The major component of oleaginous yeast and fungi is TAG composed of C_{16} and C_{18} which is similar to rapeseed and soybean oil (Madani et al., 2017). These lipophilic microbial compounds, because of their special characteristics, are considered from industrial point of view.

2.3.2 Lipid accumulation in oleaginous microorganisms

Lipid accumulation in oleaginous microorganisms occurs with starvation of nitrogen or other nutrients such as zinc, iron, phosphorus & magnesium (Kumar et al., 2020; Madani et al., 2017). Cell response to exhausting of a key nutrient is entering to lipid storage phase, in which excess carbon, convert to storage lipid. If the cells, return to a situation in which nitrogen is available, the oil reserves could be mobilized and change to cellular materials (Milano et al., 2016). Formation of lipid particles start during late exponential phase and continue during stationary phase, this process continues until carbon source in the medium start to diminish (Kumar et al., 2020; Madani et al., 2017). Lipid production in a medium with excess amount of carbon occurs in 2 stages. In the first stage cell growth occurs and this stage finishes by exhausting the nutrient except carbon. During the second phase the excess amount of carbon transform into lipid reserves. Because of nitrogen limitation, cells can no longer grow and multiply (Madani et al., 2017).

2.3.3 Fatty acid composition of single cell oil

The profile of fatty acids in oil feedstock can determine biodiesel properties such as cold flow, oxidative stability and ionic value. According to this, evaluating fatty acid composition of microbial oil is very important. Microbial oil can be converted to biodiesel by different catalysts (Kumar et al., 2020; Madani et al., 2017). Application of microbial oil as biodiesel feedstock exhibits improved fuel properties such as higher octane number and lower viscosity (Patel et al., 2020). The major fatty acid composition are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) (Chatzifragkou et al., 2011; Madani et al., 2017). The fatty acid profile is species dependent and is less infected by the type of carbon source in the medium (Xu et al., 2013). For example in *Rhodotorola mucilaginosa* the fatty acid profile is more species dependent in different carbon sources such as glucose and grass hydrolysate (Enshaeieh et al., 2012, 2013). Biodiesel typically contains methyl and ethyl esters of fatty acids. The high cost of biofuels is usually due to the high price of carbon source. Table 2.7 presents the composition of fatty acids derived from various oleaginous yeasts.

Table 2.7 Composition of fatty acids in various yeast strains.

Species	C14	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	References
Rhodotorula 110	1.11	18.51	–	1.25	67.29	4.76	Nd	(Enshaeieh et al., 2012)
Rhodotorula musilaginosa	1.98	16.62	–	1.23	69.45	5.78	0.3	(Enshaeieh et al., 2013)
Yarrowia lipolytica	-	14.8	5.9	7.5	36.5	25.8	Nd	(Fontanille et al., 2012)
Lipomyces starkeyi AS 2.1560	-	37.7	3.2	4.6	51.4	1.9	Nd	(Gong et al., 2012)
Trichosporon cunateum AS 2.571	-	27.8	0.8	20.2	48.2	3.0	Nd	(Hu et al., 2011)
Rhodosporidum toroloides Y4	-	29.8	1.1	5.9	53.2	5.8	0.7	(Zhao et al., 2011)
Cyptococcus curvatus O3	-	30.1	nd	18.5	39.3	8.3	1.2	(Zhang et al., 2011)
Rhodotorula glutinis	-	16.8	0.8	3.7	45.8	17.9	4.3	(Saenge et al., 2011)
Rhodosporidium toroloides DMKU3-TK16	0.96	22.49	-	14.56	41.54	15.12	4.51	(Kraisintu et al., 2010)
Candida curvata	nd	41.2	-	14	43	3.5	Nd	(Evans & Ratledge, 1984)

Source: Madani et al. (2017)

2.3.4 Microbial oil and biodiesel production

According to biodiesel advantages, the popularity and demand for biodiesel as an alternative for petroleum diesel is increasing (Milano et al., 2016). Limited availability and high cost of lipid feedstock for biodiesel production has led investigators to use novel lipid sources such as microbial oil. This microbial oil termed as SCO is valuable from three point of view: 1- high similarity to plants oil so can be used as biodiesel substrate. 2- lipids which are structurally similar to cocoa butter. 3- lipids that can be used as food supplements such as rare polyunsaturated fatty acids (PUFA) (Kumar et al., 2020; Madani et al., 2017). The profile of fatty acid in microbial oil is well-suited for biodiesel applications. The obtained methyl or ethyl esters can be used as biodiesel which is a good replacement for fossil fuel (Ageitos et al., 2011; Scott et al., 2011). Different vegetable oils and animal fats like soybean oil, rapeseed oil, palm oil, sunflower oil and also waste cooking oil are usually used as biodiesel feedstocks (Verma & Sharma, 2016). Due to increasing demand of biodiesel production other oil feedstock especially nonedible oils must be used. Microbial oil is one of the potential oil sources for biodiesel production. Biodiesel production is done by transesterification process. Transesterification of oils usually is done by alcohol (ethanol and methanol) and an alkali catalyst (NaOH/KOH) (Kumar et al., 2020; Madani et al., 2017). In this reaction TAG that contains fatty acids, such as palmitic, stearic and oleic acid converted to monoalkyl esters with long chain fatty acids. Transesterification involves a reaction in which the glycerol group of TAG replaces by a short chain alcohol. Enshaeieh et al. (2018) used methanol and sulfuric acid for transesterification of microbial oil from *Rhodotorula mucilaginosa*.

2.3.5 Prospects of microbial oils from various sustainable carbon sources

It has been well recognized that many oleaginous micro-organisms can convert multiple carbon sources into microbial oils. These carbon sources, however, are different in the resources, characteristics, treatment method, and fitness to oleaginous micro-organisms. Accordingly, diversified routes can be expected in order to obtain microbial oils in a more effective way. The prospects of converting various sustainable carbon sources for microbial oil production are summarized in Table 2.8, and further discussed here.

Table 2.8 Prospects of microbial oils from different sustainable carbon sources.

Carbon source	Advantages	Disadvantages, difficulties, and future efforts needed
Carbon dioxide	Reduction of CO_2 emission; low cost	Low efficiency in CO_2 utilization and conversion into microbial lipids; energy input for CO_2 supply; Smart bioreactor design and microorganism development are needed
Conventional saccharides (glucose etc.)	Effective to cell growth and lipid accumulation; utilization of energy crops at marginal lands can be considered	High cost: competition with food when starch is used as feedstock
C2 compounds	Available from a variety of biodegradable organic wastes; effective conversion was demonstrated in some oleaginous micro-organisms	Further study is needed for comprehensive demonstration of their potential applications
Conventional saccharides (glucose etc.)	Effective to cell growth and lipid accumulation; utilization of energy crops at marginal lands can be considered	High cost: competition with food when starch is used as feedstock

Source: Xu et al. (2013)

Carbon dioxide is a very attractive carbon source for microbial oil production with many expectations of reducing carbon emissions by using CO_2 waste from industries. Microalgae are widely studied because of their advantages over oil crops in higher photosynthetic efficiency, superior unit surface productivity, and simpler nutritional requirement. However, microalgae usually take at least two weeks to reach a comparable lipid production level with oleaginous yeast or fungi and have some challenges in the scale-up process. Therefore, to realize an effective microbial oil production, it is important to select and develop excellent oleaginous microalga candidates, which are able to rapidly capture and utilize CO_2, resistant to the toxic components such as SOx and NOx in the waste gases, and with good adaptability to temperature and light fluctuations. Besides, taking into account the high demand of CO_2 and water, the sites that are close to CO_2 and water resources, such as power plants, sea, and waste-water treatment plants may be suitable sites for microalgae oil production (Xu et al., 2013).

Conventional saccharides, represented by glucose, are considered as the most effective carbon resource. Effective conversion of C_2 compounds into microbial oil has been demonstrated in microalgae species like *Crypthecodinium*, *Cryptococcus*, and *Chlorella*, as well as oleaginous yeast *Candida*. Furthermore, fatty acids of microbial oils derived from C_2 compounds showed somewhat higher degree of unsaturation, which may suggest some differences in lipid synthesis. Consequently, although not sufficient in quantity, C_2 compounds can probably be used as a supporting carbon source due to their specialty in efficient carbon conversion and potential influences on fatty acid profiles. More research efforts are expected to reveal the general rules in their utilization (Verma & Sharma, 2016; Xu et al., 2013).

As the most abundant organic source, lignocellulosic biomass is undoubtedly one of the primary carbon sources for microbial oils production. Currently, the lipid production can reach 4-12 g/L after 4-10 days of liquid fermentation. The efficiency is limited mainly by the compositional complexity and heterogeneity of lignocellulosic biomass. To further improve the efficiency, it is important to select and develop strains with broad substrate utilization capability and stress resistance, and improvement in energy-saving lignocellulosic biomass pretreatment and detoxification processes will be beneficial. Furthermore, since there are few literatures that showed the natural strains being able to utilize glucose and xylose simultaneously, and targeting the issue of carbon

catabolite suppression, much work remains to be accomplished before lignocellulosic biomass become an effective carbon source for oleaginous microorganisms (Verma & Sharma, 2016; Xu et al., 2013).

Converting industrial by-products and wastes containing plenty of organic carbons into lipid is a sustainable option for recycling and conserving resources. Glycerol is a good example. As described previously, glycerol can be utilized by most oleaginous micro-organisms with similar lipid yield to that of saccharides. With the fast growing of biodiesel industries, it will be largely generated as a by-product of the transesterification process from the local plants, and thus is a promising carbon source for microbial oil production. Besides, other organic wastes, locally obtained from agriculture, fishing, or manufacturing industries, can also be useful carbon sources. However, compared with the biodiesel by-product glycerol, their complicated compositions, especially some hard-to-degrade pollutants or toxics, continue to be a challenge (Kumar et al., 2020; Madani et al., 2017; Xu et al., 2013).

2.3.6 Lipid accumulation and bioremediation of POME using microalgae

Sustainable energy management in palm oil mill has entered a new dynamic era with the chances of oleaginous microorganism culturing using POME (Chin et al., 2013; Tsang et al., 2019). Microbial treatment replacing conventional tertiary POME treatment can offer an oxygenated effluent and an ecologically safe, less expensive and more efficient mean to remove nutrients and metals. Microbial such as microalgae as a tertiary treatment nitrogen and phosphorus not removed during anaerobic digestion can reduce eutrophication at point sources better than can be achieved by conventional treatment (Cheah et al., 2018c; Reno et al., 2020). During digestion, bacteria consume the oxygen released by microalgae to decompose the organic matter, giving out carbon dioxide, ammonia, and phosphates, which are assimilated by the microalgae and methane released as energy. Sludge from wastewater treatment plant can be co-cultured with algae to enhance remediation but unlike activated sludge for of secondary effluents treatment, algae can eliminate nitrogen and phosphorus without organic carbon requirement (Ahmad et al., 2016; Kube et al., 2020). Culturing microalgae can be used as a diet supplement for fishers (Shah et al., 2018) or harvested for biodiesel (Table 2.9).

Table 2.9 The performance of biomass and lipid productions of microalgae strains cultivated in POME.

Microalgae sp.	Cultivation medium	Maximal biomass (g/L)	Growth rate (g/L) or biomass productivity* (mg/L/d)	Lipid content (%)	Lipid productivity (mg/L/d)	Nutrients reduction	References
Chlorella vulgaris	POME + 60 m/L urea	1.07	76.43	–	11.1	45.08% COD	(Nwuche et al., 2014b)
Chlorella vulgaris	40% (v/v) POME + D-glucose	1.43	–	9.7	195	–	(Nur & Hadiyanto, 2015)
Chlorella sp.	60% (v/v) POME + 80% synthetic nutrients	0.59	0.269*	7.6	11.8	–	(Nur, 2014)
Chlorella sorokiniana CY-1	30% (v/v) POME + 200 mg/L glucose + 200 mg/L glycerol	1.52	181.1	14.89	16.14	33.88% COD, 78.84 TN, 70.43% TP	(Cheah et al., 2018a)
Chlorella sorokiniana CY-1	30% (v/v) POME + 200 mg/L urea + 200 mg/L glycerol	1.38	150.0	12.53	14.41	59.25% COD, 71.38% TN, 100% TP	(Cheah et al., 2018a)

Anaerobic cultivation of *C. vulgaris* and *Scenedesmus dimorphus* with POME for 8 d HRT removes 50.5% and 86% COD; 61.6% and 86.5% BOD; and 61% and 99.5% TN, respectively (Kreith, 1999). It was previously reported that TOC of 76.6 and TN of 84% removal efficiency are achieved in the treatment of industrial wastewater by *C. vulgaris* (Choi & Lee, 2012; Zhang et al., 2012). Increasing *C. vulgaris* content from 1 to 10 g/L increase the removal rate of BOD to 80-89%, COD to 78-82%, TN to 81-85%, TP to 32-36%, NH_3-N to 99-97%, and PO_4-P to 45-49% (Choi & Lee, 2012). Shorter HRTs of 2 d have been reported for 89% BOD and 88% COD reduction using *C. vulgaris* grown in seed and animals feed production wastewater at 30 °C (Chen et al., 2008). The algae-based sewage treatment plant (STP) achieves total BOD removal of 82% (Thani et al., 1999) and 76% COD removal from piggery wastewater in high rate algal ponds (Rao et al., 2007). A study with *C. protothecoides* similarly achieves the removal efficiency of 78.3% COD, when algae is grown in concentrated soybean wastewater (Zhang et al., 2012). However, lower COD removal (59-79%) has been reported by combining the high rate algal pond, using filamentous green algae and an artificial wetland (Beccari et al., 1996).

2.3.7 Lipid accumulation and bioremediation of wastewater using yeast as inoculum

It is well established that oleaginous microorganisms including bacteria (Patel et al., 2020), yeasts (Pirozzi et al., 2015), molds (Patel et al., 2020), and algae (Dong et al., 2016) can produce microbial lipids. Among them, the most studied oleaginous microorganisms are yeast and algae due to their high cellular lipid contents. Yeasts are consider to be advantageous compared to molds and algae among the oleaginous microorganisms because of their faster growth rates and ability to be cultivated easily in large scales (Arous et al., 2016). Several oleaginous yeasts such as *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon,* and *Lipomyces* have been extensively studied for lipid accumulation. Among them, *L. starkeyi* was reported to have high flexibility in carbon source utilization and similar fatty acid composition to vegetable oils (Sreeharsha & Mohan, 2020; Vasconcelos et al., 2019).

In a recent study by Louhasakul et al. (2016), several strains of *Yarrowia lipolytica* were cultivated in POME and the strain *Y. lipolytica* TISTR 5151 produced lipid and cell-bound lipase at the highest levels of 1.64 ± 0.03 g/L and 3353 ± 27 U/L,

respectively. Moreover, the cell-bound lipase could transesterify lipid from the wet yeast cells in the direct transesterification reaction, and produced 40.90% of fatty acid methyl esters. Iwuagwu and Ugwuanyi (2014) achieved a maximum COD reduction of 83% with highest biomass accumulation in 96 h using *Saccharomyces* sp L_3^1 in POME at 150 rpm, 28 ± 2 ℃. The fungal strain *Emericella nidulans* NFCCI 3643 was proven to be an excellent biological agent in reducing the organic load of POME. The organism showed 80.28% reduction in COD, 88.23% in BOD, and 87.34% in oil/grease content at their optimal environmental and nutritional conditions (Lanka & Pydipalli, 2018a). In another study, POME degradation was succesfully carried out using *Aspergillus niger* over a period of 16 days using POME as carbon source (Loretta et al., 2016). Bioremediation of POME using indigenous yeast *Meyerozyma guilliermondii*. The remediation of POME using this strain resulted in a substantial reduction of COD of 72%, total nitrogen of 49.2%, ammonical nitrogen of 45.1%, total organic carbon of 46.6%, phosphate of 60.6%, and 92.4% removal of oil and grease after 7 days of treatment period (Ganapathy et al., 2019). In another report, the treatment of POME using *Yarrowia lipolytica* NCIM 3589, a marine hydrocarbon-degrading yeast, gave a COD reduction of about 95% with a retention time of two days (Oswal et al., 2002a). Theerachat et al. (2017) achieved a maximum COD removal of 54.7% and 48.5% from undiluted POME by *C. rugosa* and *Y. lipolytica*, respectively. Moreover, culture with *Y. lipolytica* rM-4A effectively reduced the total phenolic content in undiluted POME, resulting in removal of 36% of the total phenolic content after 96 h. Table 2.10 shows the comparison of BOD and COD removal efficacy of several microorganisms from wastewater.

Table 2.10 Comparison of BOD and COD removal efficacy of several microorganisms from wastewater.

Group	Species	Substrates	Treatment period (days)	BOD removal (%)	COD removal (%)	References
Yeast	*Yarrowia lipolytica* NCIM 3589	POME	2	77.27	95.00	(Oswal et al., 2002b)
	Y. lipolytica rM-4A	POME	6	-	48.50	(Theerachat et al., 2017)
	Saccharomyces sp L_3[1]	POME	4	-	83.00	(Iwuagwu & Ugwuanyi, 2014)
	Yarrowia lipolytica	POME	3	-	72.90	(Louhasakul et al., 2016)
	Candida rugosa CU1	POME	6	-	54.70	(Theerachat et al., 2017)
Bacteria	*Bacillus cereus* 103 PB	POME	5	90.98	78.60	(Bala et al., 2015)
	Stenotrophomonas maltophilia	POME	5	64.73	61.92	(Bala et al., 2015)
	Micrococcus luteus	POME	5	55.21	67.19	(Bala et al., 2015)
	Bacillus subtilis	POME	5	77.51	64.08	(Bala et al., 2015)
	Bacillus cereus	Petroleum wastewater	28	-	70.00	(Agarry, 2017)
	Rhodococcus opacus	Dairy waste	4		65.00	(Kumar et al., 2015)
	Bacillus cereus	Unhairing wastewater	10	-	41.00	(Mlaik et al., 2015)
	Bacillus cereus GS-5	Domestic wastewater	13	-	90.60	(Rout et al., 2018)
	Bacillus cereus MTCC 25641	Dairy Waste Effluent	7	44.07	50.68	(Gawai et al., 2017)
	Bacillus licheniformis	Municipal wastewater	4	72.08	51.00	(Garode, 2018)
	B. cereus KM15	Orange waste	7		62.80	(Qadeer et al., 2018)

2.3.8 Lipid accumulation through bioremediation using bacterial inoculum

Bacteria generally accumulate polyhydroxyalkanoates (PHAs), mainly polyhydroxybutyrate (PHB), for energy storage; TAGs are only produced by some strains (Kosa & Ragauskas, 2011). Bacteria can be more advantageous in lipid accumulation over other oleaginous microorganism due to have a less complex genome, metabolism, and cell compartmentation and can produce a wider range of different types of lipids compared to multicellular eukaryotes. Moreover, it is an aerobic microorganism with easier cultivation, diverse carbon sources and carbon utilization pathways, natural ability to store lipids and PHA as reserve food material, balance fuel properties composition (Kumar et al., 2018). In addition, they can usually be genetically modified more readily to obtain optimized strains that further increase the productivity and competitiveness of the whole process (Chisti, 2007; Kosa & Ragauskas, 2011; Rude & Schirmer, 2009). Furthermore, it was also reported that bacteria was very efficient to utilize a wide range of waste substrate as carbon source such as urban wastewater (Cea et al., 2015), milk processing wastewater (Cea et al., 2015), POME (Zhang et al., 2016), mango waste, orange waste, apple waste (Qadeer et al., 2018), dairy waste (Kumar et al., 2015), molasses (Kumar et al., 2015), carob waste, orange waste (Gouda et al., 2008), cotton stalk (Zhang et al., 2014b), POME (Bala et al., 2018). Until now, quite a few studies have reported the capability of bacteria in lipid accumulation (Meng et al., 2009); but few studies have explored that several species of bacteria such as *Rhodococcus opacus*, *Pseudomonas* spp., *Bacillus cereus* and *Bacillus subtilis* were able to accumulate a significant amount of lipids as TAGs (Brigham et al., 2011; Kumar et al., 2018). In a recent study, Qadeer et al. (2018) obtained a removal of VS up to 38.5% and oxidizable organic matter removal (COD-based) up to 48.9% through the degradation of mango waste by using *B. cereus* KM15, while simultaneously showing an accumulation of lipids up to 41.5% in 96 h. The degradation efficiency of organic matter was 30.9 and 31% for apple and orange waste after 96 h with a lipid accumulation of 21 and 25%, respectively. Table 2.11 shows the performance of oleaginous yeasts and bacteria to produce microbial lipid from POME.

Apart from the lipid production, the bacterial strains, especially, the strains of *Bacillus*, could significantly remediate pollutants from the wastewater, as they are

Table 2.11 Performance of oleaginous yeasts and bacteria to produce microbial lipid from wastewater.

Group	Microorganisms	Culture conditions	Biomass, g/L	Lipid, g/L	Lipid content, (wt.%)	References
Yeast	*Yarrowia lipolytica*	two-fold diluted effluent	3.79 ± 0.04	1.15 ± 0.11	30.34 ± 1.45	(Louhasakul et al., 2016)
	Yarrowia lipolytica	un-diluted effluent nitrogen source	5.68 ± 0.32	1.64 ± 0.03	28.87 ± 0.26	(Louhasakul et al., 2016)
	Candida silvae	POME (90%v/v)	11.71 ± 0.8	1.85	15.81 ± 1.9	(Marjakangas et al., 2015)
	Galactomyces geotrichum	POME (90%v/v)	10.92 ± 0.5	0.81	7.42 ± 1.4	(Marjakangas et al., 2015)
	Lecythophora hoffmannii	POME (90%v/v)	13.01 ± 0.5	1.22	9.42 ± 1.3	(Marjakangas et al., 2015)
	Graphium penicillioides	POME (90%v/v)	12.91 ± 0.1	2.37	18.41 ± 1.0	(Marjakangas et al., 2015)
Bacteria	*Pseudomonas* sp.	POME (30% v/v)	1.91 ± 0.07	0.31 ± 0.04	16.04	(Zhang et al., 2016)
	Bacillus sp. V10	milk processing wastewater 48h,	1.50	0.09	6.10	(Cea et al., 2015)
	B. cereus KM15	apple waste, 3 days	25.00	7.50	30.00	(Qadeer et al., 2018)
	Rhodococcus opacus DSM 43205	dairy waste, 4 days,	3.71	1.89	51.00	(Kumar et al., 2015)
	Bacillus subtilis HB1310	cotton stalk, 2 days	5.70	2.30	39.80	(Zhang et al., 2014b)
	Rhodococcus opacus	Dairy wastewater	4.00	3.16	79.00	(Gupta et al., 2018)
	Gordonia sp. DG	Orange waste	0.12	0.06	50.00	(Gouda et al., 2008)

capable of excreting different types of enzymes such as cellulase, laccase, and lipase which would promote waste digestion (Bala et al., 2014). Furthermore, *Bacillus* possess a higher tolerance of environmental fluctuations such as low pH, high temperature due to their spore forming capability (Grady et al., 2011). In a recent study, *B. subtilis* was able to synthesize a lipid content of 39.8% in 48 h, when cultured in cotton stalk hydrolysate as substrate (Zhang et al., 2014b). Bala et al. (2015) reported a higher reduction of COD (78.60%) from POME by using a strain of *Bacillus cereus* 103 PB compared to other bacterial strains, such as *Micrococcus luteus* 101 PB (67.19 %), *Stenotrophomonas maltophilia* 102 PB (61.92 %), *Providencia vermicola* 104 PB, *Klebsiella pneumoniae* 105 PB and *Bacillus subtilis* 106 PB (64.08 %). Bhumibhamon et al. (2002) has reported removal of fat and oil by *Bacillus* sp. KUL39 (81.6%) from wastewater of palm oil and bakery industries. Nanganuru et al. (2012) reported 71% removal of oil by *B subtilis*. Marina et al. (2013) has also reported treatment of oily wastewater with *B. cereus*. In another study by (Banerjee & Ghoshal, 2017), petroleum wastewater samples from oil refinery and oil exploration site were treated by hyper phenol-tolerant *B. cereus* (AKG1 and AKG2) in laboratory-scale batch process to assess their bioremediation efficacy. In another study, POME degradation was successfully carried out using *B. subtilis*, *P. aeruginosa* over a period of 16 days using POME as carbon source (Loretta et al., 2016). Simultaneous lipid production and dairy wastewater treatment using *Rhodococcus opacus* in a batch bioreactor for potential biodiesel application was performed by Kumar et al. (2015). The bacteria accumulated 14.28% w/w lipid and reduced the initial wastewater COD by 30% using the raw dairy wastewater.

2.3.9 Microbial co-culture for enhanced lipid production

Lipid production performance could be significantly increased by using different technique such as metabolically engineered strain; however, the proliferation and stability of modified microbes deserves to be further studied. Microbial co-culture in wastewater could be more efficient culture strategy to increase the productivity and decrease production costs. Recently, Cheah et al. (2018c) demonstrated a satisfactory biomass and lipid yield by using co-culturing of bacteria (i.e., *Pseudomonas* sp.) on microalgae (i.e., *C. sorokiniana* CY-1) through an effective POME bioremediation. Cheah et al. (2018c) obtained a satisfactory biomass growth of 2.04 g/L with a productivity of 185.71

mg/L/day and lipid content (16.04%) by co-culturing bacteria (i.e., *Pseudomonas* sp.) on microalgae (i.e., *Chlorella sorokiniana* CY-1) through an effective POME bioremediation (COD removal). In another study, Bala et al. (2015) found that the reduction efficiency of COD (90.64%) by bacterial co-culture (*B. cereus* 103 PB and *B. subtilis* 106 PB) was significantly higher than the mono-culture. Moreover, the reduction efficiency of COD for the bacteria combination *B. cereus* 103 PB and *B. subtilis* 106 PB (90.64 %) was higher than *Micrococcus luteus* 101 PB and *Stenotrophomonas maltophilia* 102 PB (71.84 %) and control (14.35 %) (Bala et al., 2015). Nevertheless, a mixed culture of microalgae (*Chlorella vulgaris*) and yeast (*Rhodotorula gultinis*) produced a higher biomass (4.63 g/L) and lipid (2.88 g/L) than that in the pure cultures (Cheirsilp et al., 2011).

The wastewater treatment utilizing the algal-bacterial system is capable of removing about 80% COD (Weiland, 2010). Utilizing *N. oculata*, and *Chlorella* sp. the highest removal of COD (95-98%), BOD (90-98%), TOC (80-86%) and TN (80%) were achieved after 7 d of anaerobic treatment as compared to treatment without microalgae (Ahmad et al., 2014a, 2015). POME treated with anaerobic co-cultivation of *Tetraselmis suecica* achieved high removal efficiency of COD, BOD, TOC and TN after 3 and 7 d of HRT at 87-95%, 84-95%, 67-90%, 73-80%, respectively (Ahmad et al., 2014b). The lower removal efficiency of COD (53%), BOD (73%), TOC (49%) and TN (48%) are achieved on day 3 of aerobic treatment without microalgae. Filtered POME composition in sea water at different levels (1, 5, 10, 15 and 20%) used as an alternative medium produced enhanced cell growth and lipid accumulation at 10% POME for *N. oculata* and *T. suecica* with maximum specific growth rate (0.21/d and 0.20/d) and lipid content (39% and 27%), respectively, after 16 d of flask cultivation. The POME/Seawater media with algal treatment had been observed to achieve significant removal COD (93.6-95%), BOD (96-97%), TOC (71-75%), TN (78.8-90.8%) and oil and grease (92-94.9%). The major fatty acids composition of lipid recovered from *N. oculata* and *T. suecica* cultivated in 10% POME composition with sea water are pentadecanoic acid (C15:0), palmitic acid (C16:0), stearic acid (C18:0) belonging to saturated fatty acids (SFA); and palmitolic acid (C16:1) and oleic acid (C18:1) belonging to monounsaturated fatty acids (MUFA). The total SFA (59.24%, 68.74%); MUFA (15.14%, 12.26%); and PUFA (9.07%, 8.88%) are obtained for *N. oculata* and *T. suecica*, respectively. *N. oculata* contained high palmitic acid (C16:0) at 28.22% and palmitolic (C16:1) at 9.37%

while *T. suecica* contained high palmitic acid (C16:0) at 36.48% and pentadecanoic acid (C15:0) at 9.21%. In PUFA profile, the highest percentage of linolenic acid (C18:3) is found in *N. oculata* (4.54%) and *T. suecica* (5.11%). The cultivation of *N. oculata* and *T. suecica* in 10% POME composition with sea water therefore is suitable for cell growth as well as MUFA and PUFA production. The percentage of fatty acids content of microalgae can be tuned based on the growth phases from which the cultures are harvested. With high saturated and monounsaturated fatty acids, *N. oculata* and *T. suecica* are potential candidates for the production of biodiesel (Shah et al., 2016). Table 2.12 the performance of several co-culture inoculums to produce microbial lipids and COD removal from wastewater.

Theerachat et al. (2017) observed that the highest removal of triglyceride (98.5%) and COD (60.3%) can be obtained by the co-culture of *C. rugosa* and *Y. lipolytica* in the undiluted POME for 120 h. A mixed culture oleaginous yeast *Rhodotorula gultinis* and microalgae *Chlorella vulgaris* produced higher biomass (4.63 g/L) and lipid (2.88 g/L) than that in the pure cultures (Cheirsilp et al., 2011). The synergistic effect of yeast-bacteria co-culture on bioremediation of oil-contaminated soil was studied by (Zhang et al., 2014a), and they removed 56% of total petroleum hydrocarbon and 32% of polycyclic aromatic hydrocarbons. Islam et al. (2018a) achieved a maximum power density and higher COD removal efficiency in microbial fuel cell by using mutualistic interaction of yeast and bacteria (*L. starkeyi* and *Klebsiella pneumonia*). However, the combination of yeast and bacteria and their interactions in accumulating microbial lipids through bioremediation of POME have not reported to date. Lipid production and simultaneous COD removal efficiency could be enhanced through manually adjusting the microbial cooperation. Especially, when organic waste/wastewater used as substrate, degradation process can be more complex. In this case, inoculating strains according to the substrate can help to reduce the adaptation time and enhance the lipid production. It was hypothesized that *B. cereus* would boost up the growth and the lipid accumulation performance of *L. starkeyi* by quick assimilation of organics present in the POME having a higher degradation efficiency.

Table 2.12 Performance of co-culture to produce microbial lipids and COD removal from wastewater.

Strains	Co-culture consortia	Substrates/treatment period	Biomass (g/L)	Lipid (g/L)	Lipid content (wt%)	COD removal (%)	References
Pseudomonas sp. on *C. sorokiniana* CY-1	Microalgae-bacteria, 1:1	30% (v/v) POME, 5 days	2.04	0.33	16.04	53.70	(Cheah et al., 2018c)
Klebsiella variicola and *P. aeruginosa*	Bacteria-bacteria, 1:1	50% (v/v) POME, 11 days	-	-	-	69.28	(Islam et al., 2018a)
B. cereus 103 PB and *B. subtilis* 106 PB	Bacteria-bacteria, 1:1	POME, 5 days	-	-	-	90.64	(Bala et al., 2015)
R. gultinis and *C. vulgaris*	Yeast-microalgae, 1:1	Sugar cane plant wastewater (molasses), 7 days	4.63	2.88	-	79.00	(Cheirsilp et al., 2011)
Scenedesmus obliquus with *Pseudomonas* sp.	Microalgae-bacteria, 2:1	BG11 medium, 10 days	2.96	0.68	21.10	-	(Wang et al., 2015a)
Rhizobium radiobacter and *C. vulgaris*	Bacteria-microalgae, 0.3	BG11 medium, 12 days		-	25.80	-	(Wang et al., 2015b)
C. rugosa CU1 and *Y. lipolytica* rM-4A	Yeast-yeast, 1:1	50% (v/v) POME	-	-		60.30	(Theerachat et al., 2017)

2.4 Optimization of culture conditions for enhancing lipid production performance

2.4.1 Overview of optimization

Optimizing the operational parameters in microbial lipid accumulation and bioremediation could enhance the efficiency of the operation and simultaneously reduce the energy and time. Recently, in biological operating systems, statistical optimization has emerged as a popular technique due to the increasing impact of parameters. In addition, this optimization technique can be employed to search within a wide experimental area with the least number of runs, and can also provide the contribution of each factor and its share on the different responses (Rajendhran et al., 2002). The interactions among different variables can also be evaluated. The influence of certain factors can be analyzed at different levels of other factors, thus, the conclusions are more accurate over the total experimental space (Madani et al., 2015; Subhash & Mohan, 2014).

Lipid accumulation and fatty acid composition of oleaginous microorganisms varies depending on environmental factors (such as pH, temperature and incubation time) and the nature of the microorganism (Ageitos et al., 2011; Subhash & Mohan, 2014). 'One variable at a time' is the classical method of approach that permits the determination of specific requirements for growth and product formation by systematically adding or deleting components from the medium (Abdelhamid et al., 2019). A considerable number of works have been done to study the effect of operational parameters in microbial lipid accumulation performance. Such studies have covered operational areas like initial medium pH, incubation temperature, incubation period, inoculum size, static and shaking conditions, different carbon sources, different nitrogen sources (Abdelhamid et al., 2019; Ali et al., 2017; Ali & El-Ghonemy, 2014). 'Mathematical modelling' is considered as a powerful tool for investigating the effect of the aforementioned aspects on the overall performance of lipid accumulation and bioremediation (Dai et al., 2011; Ortiz-Martínez et al., 2015; Pinto et al., 2012; Wen et al., 2009). The use of mathematical models could provide deeper insight in terms of the analytical measurement of the state hard-to-measure aspects, and thereby, reduce the analysis effort. The mathematical models can describe the conversion of a complex systematic phenomenon due to its versatility and that enables relatively simple series of mathematical expressions to describe the influence of each element on the total output (Ortiz-Martínez et al., 2015). Generally, mathematical

modelling is accomplished in two approaches; one approach is to derive the model based on the physical or engineering laws that govern the system processes (Joseph & Melkote, 2009; Joseph & Yan, 2015). But, most engineering models are called deterministic models since the parameter values and the initial conditions determine the output of the model (Thomann, 1989).

The second approach is the statistical tools which are designed based on the data collected during the fermentation process (Ali et al., 2017; Joseph & Yan, 2015; Shoaib et al., 2018), and are important for evaluating the relationships between the system inputs and outputs means the relationship between the culture conditions/nutritional factors and lipid production, particularly when there is a limited engineering domain knowledge to be characterized, such as the complex mechanisms of lipid production and organic compound removal (Subhash & Mohan, 2014). Furthermore, statistical models can efficiently capture the system ambiguity and remedy the error created from engineering models for a better system quantification (Joseph & Melkote, 2009). In general, the operational parameters such as pH, temperature, substrate concentrations, incubation time, and different nutritional factors (Abdelhamid et al., 2019; Ali et al., 2017; Ali & El-Ghonemy, 2014). Recently, response surface methodology (RSM) has been extensively applied in the optimization of medium composition and culture conditions (Awad et al., 2011). RSM is a collection of mathematical and statistical techniques for the experimental design, evaluation factors, model development and optimum conditions of different biotechnological processes. Statistical optimization not only allows quick screening of large experimental domain but also reflects the role of each component (Ali et al., 2017).

2.4.2 Effect of operational parameters on lipid production

Various strains of several oleaginous microbes could accumulate significant amount of lipids but not all of them may be able to efficiently utilize the nutrients from POME. Extensive research was carried out on the development of microbial oil production through the cultivation of oleaginous microorganisms in the POME (Subhash & Mohan, 2014). Several operational parameters such as pH, substrate concentrations, inoculum compositions, temperature, and time severely influence the performance of fermentation process for bioremediation and lipid accumulation. It was observed that the lipid content and fatty acid composition varies depending on the nature of microorganism and the environmental and nutritional culture conditions, such as substrate type and

concentration, medium pH, incubation temperature, static and shaking condition, nutrients (especially, carbon and nitrogen sources, C/N ratio), and the inoculum composition (Ali & El-Ghonemy, 2014; Subhash & Mohan, 2014). For instance, Abdelhamid et al. (2019) reported that medium pH is one of the most important environmental factors influencing bacterial cell growth and physiology; therefore, plays an important role in the growth and lipid accumulation. Indeed, lipid accumulation is greatly influenced by the growth of microbes and C/N ratio in the medium because lipid accumulation occurs in nitrogen limiting condition. Nevertheless, the microbial growth is dependent to the operating parameters and nutrients. Higher C/N ratio and organic nitrogen sources have been shown to favor an oil accumulation in microbial biomass (Huang et al., 2010). Subhash and Mohan (2014) studied the influence of several operating parameters including pH, temperature, glucose, nitrogen, phosphorous, proteins and sodium chloride concentration on fungal growth and lipid accumulation; and they found that pH, glucose, incubation temperature, and incubation time substantially influenced the lipid production. Wang et al. (2015b) demonstrated that inoculation ratio, initial glucose concentration, and co-culture time were the most significant process variables in the co-culture of bacteria and microalgae. A mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* showed highest biomass (of 4.63 ± 0.15 g/L) and lipid production (of 2.88 ± 0.16 g/L) after five days of cultivation in an industrial waste (steamed fish processing effluent) at optimal conditions of inoculum ratio of yeast to microalga at 1:1; initial pH at 5.0; molasses concentration at 1%; shaking speed at 200 rpm; and light intensity at 5.0 klux under 16:8 hours light and dark cycles (Cheirsilp et al., 2011).

2.4.2.1 Effect of inoculum composition

In a recent study by Abdelhamid et al. (2019), the inoculum size of *Penicillium commune* NRC2016 was adjusted at different values. The media pH was adjusted at 7.0 then incubated for 5 days at a temperature of 20 °C. The maximum lipid production reached 30.37% and was obtained by using inoculum size 0.75% while the minimum value reaches to 28.50% by using inoculum size 0.50%. Therefore, it can be said that the inoculum composition significantly influences on the microbial growth and lipid accumulation. Recently, the co-culture inoculums have gained attention to the researchers for enhancing lipid production, because the collective output of co-culture is usually

higher than that of the monoculture systems (Islam et al., 2018a; Kim et al., 2016; Venkataraman et al., 2011). The inter-microbial interactions within the microbes play a crucial role to determine evolutionary relationship between or within the microbes in a microbial community, along with system properties, particularly the dynamics and stability of the entire microbial communities. In the past few years, the use of synergistic microbial consortia has renewed attention because the synergistic interaction between microbes enabled degradation capability in wide range substrates and could enhance the lipid accumulation as well (Islam et al., 2019). In general, the microbial interactions could be neutral (Read et al., 2010), while in some cases it could be synergistic (Venkataraman et al., 2011), and or antagonistic (Powers et al., 2015).

The synergistic co-culture inoculums are preferred because the combined output through mutualistic interactions is usually higher than that of the monoculture systems cycles (Cheirsilp et al., 2011). However, it should be noted that the ratio of microbes plays a crucial role, especially in the synergistic interactions which occur in the co-culture-inoculated fermentation process (Cheah et al., 2018c). For instance, the higher biomass (2.04 g/L) and productivity (185.71 mg/L/d) were attained by co-cultivation of *C. sorokiniana* CY-1 and *Pseudomonas* sp. with a ratio of 1:1 in the POME. At this inoculum ratio, lipid content (16.04%) was about two fold higher than other ratios of 2:1 or 1:2. Similarly, Cheirsilp et al. (2011) obtained higher lipid production (2.88 ± 0.16 g/L) and COD removal (79.0 ± 1.1%) using a co-culture of microalga *C. vulgaris* and yeast *R. glutinis* in the ration of 1:1 (Cheah et al., 2018c). In another study, the ratio of 0.20–0.25 for bacteria and microalgae was observed as optimum culture conditions to obtain maximum lipid accumulation (Wang et al., 2015b). Although several studies have been reported on the synergistic effects on co-culture inoculum in lipid accumulation, none have reported the effect of inoculum ratio and their interaction with other process parameters on the performance of lipid production and COD removal. Therefore, this study provides information on the influence of co-culture inoculum ratio on the performance of lipid production and COD removal efficiency.

2.4.2.2 Effect of pH

Generally, the microbes require a pH close to neutral for their optimal growth. It is well known that most bacteria can grow well around pH value of 6.5-7.0, however,

each microbial species has its own optimum pH range for best growth. Moen et al. (2003) reported that the optimum pH for methanogenic archaea was 6.6–7.5, while Yuan et al. (2015) observed that the dominant bacterial genus responsible for fermentation usually grows at a pH range of 4 to 10. For instance, microorganisms such as *Clostridium*, *Ruminococcaceae*, *Actinomyces*, *Peptostreptococcaceae*, *Tetrasphaera*, and *Zoogloe* grow well at pH 4, while *Alcaligenes*, *Anaerolinea*, *Paludibacter*, and *Tissierella* grow well at or near pH 10. Jones et al. (2015) reported the optimum pH range of 6.6-7.0 for *P. aeruginosa*, whereas *Klebsiella* grows better in the pH range of 5.5 -7.0. Zhao et al. (2017) showed that the maximum growth of *Lactobacillus* bacteria was at initial pH of 6.5, however, pH below 5.0 was not favorable for the growth of bacteria. In the case of yeast, the growth was maximum at pH 6.0, but similar growth was observed at pH 5.5 (Zhao et al., 2017). Therefore, it is concluded that the neutral pH is imperative to achieve optimal microbial growth as well higher COD removal and lipid accumulation.

The initial medium pH was found to be a significant factor for lipid accumulation. (Lilly & Barnett, 1951) recorded that the hydrogen ion concentration in the medium was an influential factor for growth and other life processes like sporulation. It was known that the function of plasma membrane was to regulate the transport of substances from in and out the cells. Previous studies recorded the influence of pH value on the microorganism's growth kinetics and concluded that the medium pH was an important environmental factor affecting cell growth and products formation (Abdelhamid et al., 2019). Comparable results were obtained by Ruan et al. (2014), Ali and El-Ghonemy (2014), and Jiru et al. (2017); they recorded that pH values between 5 and 6 were found to be the suitable pH for most fungal growth. Therefore, the ratio of co-culture inoculum composition can be severely affected by varying the medium pH and that, in turn, influences the performance of fermentation.

The effect of pH on the lipid production by *Aspergillus wentii* Ras101 was studied in the recent research work by Shoaib et al. (2018). This result reveals that the lipid production is increased with increasing the pH. The optimized value obtained from LINGO optimization program is about 6.1 at which the optimum value of the produced lipid is 40% of the obtained dry biomass. Minhas et al. (2016) reported that the pH of the fermentation medium is an important environmental factor affecting cell growth and products formation. Lilly and Barnett (1951) reported that the hydrogen ion concentration

of the culture medium is strongly affecting the growth, sporulation, and metabolic activities of fungus. Besides, the external pH of the medium may affect the plasma membrane permeability; consequently, the change of the external pH affects the membrane osmosis towards the absorption of the different ions and nutrients from the surrounding medium (Amanullah et al., 2001).

2.4.2.3 Effect of incubation temperature

The incubation temperature had an impact on the biomass formation and lipid synthesis. In a recent study, Abdelhamid et al. (2019) demonstrated that the incubation temperature significantly influenced the lipid accumulation and the maximum lipid accumulation for *Penicillium commune* NRC2016 reached 41.18% at 20 °C similar to that of Carlile et al. (2001) who investigated that all fungal enzymes exhibited high activity at a temperature 20-30 °C. In another study, Ali et al. (2017) showed that there was an increase in growth biomass as well as lipid accumulation with the increase of incubation temperature from 25 °C to 35 °C or for *P. brevicompactum* NRC 829. However, the highest lipid productivity was reported at 30 °C (39.0 ± 1.43% lipid/dry biomass) during the preliminary screening, while a decrease in biomass and lipid accumulation was noticed with lower/higher incubation temperature compared with the optimum temperature. These results are in accordance with those reported by Ali and El-Ghonemy (2014) and Subhash and Mohan (2014). In this regard, Carlile et al. (2001) reported the optimum temperature for maximum fungal biomass to be 30 °C, which might be attributed to the natural environments of fungi. At high temperatures, an increase in nutritional requirements is sometimes observed in Saccharomyces (Carlile et al., 2001).

The effect of incubation temperature on lipid production by *A. wentii* Ras101 was also reported by Shoaib et al. (2018). Incubation temperature was one of the most important factors which affect the lipid accumulation. Therefore, the fungal strain (*A. wentii* Ras101) was incubated at varying temperatures ranging from 15 to 35 °C (Liu et al., 2010). As reported in the study of Shoaib et al. (2018) showed that, lipid content increases with increasing the incubation temperature until it is reached the value of 28 °C. At incubation temperatures higher than 28 °C, the lipid production is decreased. The optimum temperature achieved by Lingo program was 28 °C, which corresponds well with the experimental value.

2.4.2.4 Effect of incubation time

The time of incubation is also an important factor and showed an influence on the microbial biomass formation and lipid accumulation. Lipid production of each strain differs depending upon the specific growth rate of the strain, whereas the maximum lipid production could be obtained only after a certain incubation time which allows the culture to grow at a steady state. In the current study, the highest biomass and lipid accumulation were noticed on the 6th day of incubation (39.5 ± 1.28% lipid/dry biomass) *P. brevicompactum* NRC 829 (Ali et al., 2017). In contrast, the highest biomass and lipid accumulation of *Rhodosporidium toruloides* and *Trichoderma viride* were reported on the 5th day of incubation (Ali & El-Ghonemy, 2014). On the other hand, El-Fadaly et al. (2009) reported 2.2 g/L of microbial oil with 59.5% oil percentage after 72 h of incubation from *Cryptococcus curvatus*. They suggested that, after inoculation, up to 72 h the fungus consumed all the available nitrogen, and then the reserve lipid was synthesized in distinct oil droplets. In a recent study, Abdelhamid et al. (2019) demonstrated that the incubation time had a significant effect on lipid production by *P. commune* NRC2016 and maximum lipid production reached 46.36% after the fifth day. The result was similar to Ali and El-Ghonemy (2014) for *Aspergillus* sp. and *T. viride* NRC314 and reported that maximum lipid production was obtained after incubation time of 5 days.

Shoaib et al. (2018) also demonstrated that the incubation time is also an important factor for the fungal strain growth and biomass formation using *A. wentii* Ras101. The determination of the optimum incubation time can guarantee the efficient utilization of available carbon source in the growth medium, which leads to a higher accumulation of lipid content. According to their observation, the lipid production was increased when the incubating time was increased up to about 170 h. The optimum time required for the fungal strain growth was about 168 h (7 days). These results was in agreement with data obtained by Ali et al. (2017), who reported that the maximum lipid production by *Aspergillus* spp. was obtained after 5 days of incubation.

2.4.3 Importance of optimization of process parameters in lipid production

Difference in several culture conditions greatly affect the lipid accumulation ability of microbes as well as the fatty acid composition. Zhao et al. (2017) showed that the maximum growth of *Lactobacillus* bacteria was at initial pH of 6.5, however, pH

below 5.0 was not favorable for the growth of bacteria. As to *Bacillus subtilis*, the bacteria reproduced well at pH 6.0-8.0. In the case of yeast, the growth was maximum at pH 6.0, but similar growth was observed at pH 5.5. They also stated that the optimum temperatures for both bacteria and yeast were observed at 28-32°C when they were cultured at their optimal pH values. Shoaib et al. (2018) reported that the lipid accumulation by a fugal strain *A. wentii* Ras101was increased with increasing the pH up to 6.1, then gradually decreased. They also stated that the lipid content increases with increasing the incubation temperature until it is reached the value of 28 °C and gradually decrease at incubation temperatures higher than 28 °C. In a recent study by Subhash and Mohan (2014), a pH 5.5 and temperature of 30 ℃ were found to be suitable for growth and lipid accumulation by oleaginous fungus *A. awamori*. Nevertheless, the lipid production was increased when the incubating time for the fungal strain growth was increased up to about 168 h (7 days), and then slightly deceased (Shoaib et al., 2018). Ali et al. (2017) reported that the maximum lipid production by *Aspergillus* spp. was obtained after 5 days of incubation. Higher biomass growth and lipid accumulation was noticed at 72 h for oleaginous fungus *A. awamori* (Subhash & Mohan, 2014). In a mixed culture of oleaginous yeast *R. glutinis* and microalga *C. vulgaris* showed highest biomass and lipid production after five days of cultivation in an industrial waste, then slightly decreased in lipid production on day 7 (Cheirsilp et al., 2011). Furthermore, inoculum ratio was found to have a significant influence on the biomass growth and lipid accumulation in co-culture cultivation. *Pseudomonas* sp. was co-cultivated with *C. sorokiniana* CY-1 in ratios of microalgae versus bacteria of 1:1; 2:1 and 1:2. Higher biomass (2.04 g/L) and productivity (185.71 mg/L/d) were attained in the ratio of 1:1. At this inoculum ratio, lipid content (16.04%) was about two fold higher than other ratios (Cheah et al., 2018c). In another study, the optimized culture conditions of inoculation ratio of bacteria and microalgae of 0.20–0.25 was observed to be effective inoculum for lipid accumulation (Wang et al., 2015b). Therefore, it can be said that the operating parameters have a significant influence on the microbial growth and lipid accumulation. In this context, the optimization of cultivation conditions could be favorable to maximize lipid production by oleaginous microorganism, particularly, in the case of co-culture inoculum.

2.4.4 Statistical models used in lipid accumulation or bioremediation

Statistical models are constructed based on the data derived from the fermentation process. The statistical models are suitable for revealing the relationships between inputs and outputs, and for determining the significant parameters for system quantifications, particularly when engineering models are difficult to construct. The ambiguity in the system can also be quantified using statistical models rather than engineering models. However, the conclusions made from the statistical models need to be validated with engineering knowledge. A wide variety of statistical models ranging from regression-based methods to data mining methods, can be applied for fermentation system to enhance lipid accumulation and bioremediation performance, as presented in Table 2.13.

The effect of different operational parameters, such as pH and substrate concentration on the performance of lipid accumulation and COD alteration efficiency have been studied using factorial design (FD), central composite design (CCD), and analysis of variance (ANOVA)-based-RSM (Abdelhamid et al., 2019; Ali et al., 2017; Madani et al., 2015). It was observed that an increase in substate concentration could lead to an increase in the performance of lipid accumulation at a moderate pH level, while a contrary effect could be observed at a low pH level. However, the interactions between/among parameters cannot be determined using an one factor at a time (OFAT) model (Abdelhamid et al., 2019; Madani et al., 2015). Optimization of process parameters can be performed using OFAT, CCD, Box-Behnken design (BBD), FD, and Placket-Burman design (PBD). The factor setting for maximum performance is obtained and validated by real experiments (Madani et al., 2015; Shoaib et al., 2018; Subhash & Mohan, 2014).

Table 2.13 Summary of statistical models used in lipid production by different microorganisms.

Methods/Tools	Data Generation	Microorganisms	Major conclusion	References
RSM	CCD	*Penicillium brevicompactum* NRC 829MFC	Temperature, pH, time, concentration of $NaNO_3$, and KCl were significant	(Ali et al., 2017)
DOE using Taguchi's approach	Orthogonal array (OA)	*Aspergillus awamori* (MTCC11639)	pH and carbon source factors were significant in lipid production	(Subhash & Mohan, 2014)
SLR	OFAT	*Penicillium commune* NRC2016	pH, time, and temperature were significant	(Abdelhamid et al., 2019)
ANOVA	Duncan's multiple range tests	*Rhodotorula glutinis* and *Chlorella vulgaris*	pH, agitation speed, and incubation time were significant	(Cheirsilp et al., 2011)
RSM	LINGO/ NLP	*Aspergillus wentii* Ras101	Medium composition, initial pH, incubation temperature and incubation period were the main factors	(Shoaib et al., 2018)
ANOVA	FD	*Nannochloropsis oculata* and *Tetraselmis suecica*	Substrate concentration and incubation time were significant	(Shah et al., 2016)
RSM	FD, CCD	*Chlorella vulgaris* and *Rhizobium radiobacter*	Inoculation ratio of bacteria and microalgae, initial glucose concentration, and co-culture time were significant variables	(Wang et al., 2015b)

The effect of different operational parameters, such as pH and substrate concentration on the performance of lipid accumulation and COD alteration efficiency have been studied using factorial design (FD), central composite design (CCD), and analysis of variance (ANOVA)-based-RSM (Abdelhamid et al., 2019; Ali et al., 2017; Madani et al., 2015). It was observed that an increase in substate (glucose) concentration could lead to an increase in the performance of lipid accumulation at a moderate pH level, while a contrary effect could be observed at a low pH level. However, the interactions between/among parameters cannot be determined using an one factor at a time (OFAT) model (Abdelhamid et al., 2019; Madani et al., 2015). Optimization of process parameters can be performed using OFAT, CCD, Box-Behnken design (BBD), FD, and Placket-Burman design (PBD). The factor setting for maximum performance is obtained and validated by real experiments (Madani et al., 2015; Shoaib et al., 2018; Subhash & Mohan, 2014). Several types of models, including CCD, FD, UD and BBD have been used to study the effect of factors on lipid accumulation, as presented in Table 2.13.

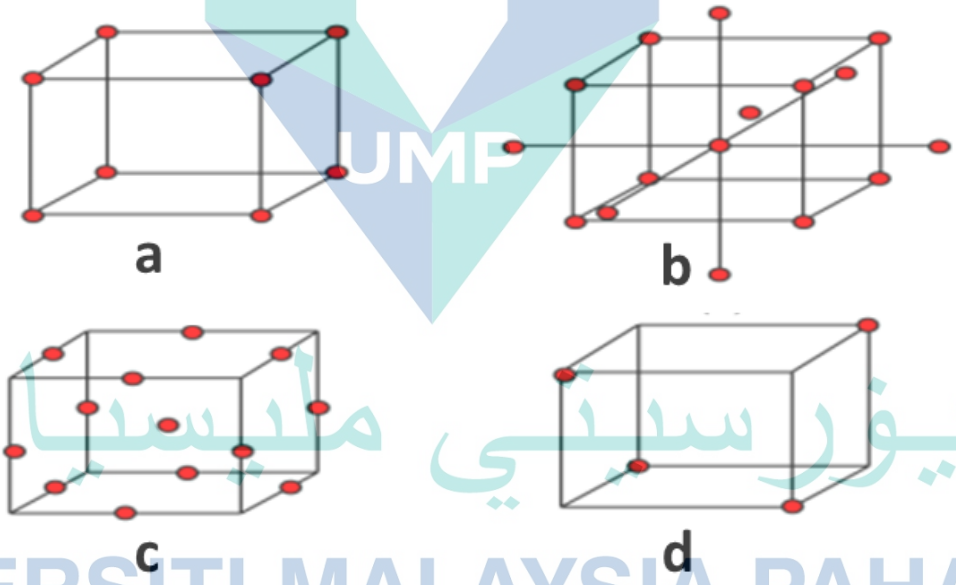

Figure 2.23 Design of experiments used in the different studies, a) factorial design, b) central composite design, c) Box-Behnken design and d) Placket-Burman design

Source: Luo et al. (2016)

Figure 2.23 demonstrates some of the designs for a three-factor problem, where the red points show the settings of the experimental runs, while the cube depicts the space of the experimental design. In the FD, only the linear effect of factors can be estimated even though it takes two levels of values (Figure 2.23a), but in the BBD and CCD, the quadratic effect can be estimated without introducing additional experimental runs since

the factors take more levels of values (Figure 2.23b-c). However, PBD requires a limited number of experiments to screen a relatively large number of factors (Figure 2.23d). In the initial screening, the interactions between the factors are usually ignored. For the UD, experimental points are uniformly allocated in the domain and are used when the underlying model structure is completely unknown (Jia et al., 2014). It is worthy to note that other related but un-controlled factors can be encountered when performing DOE-guided experiments; these factors are usually denoted as co-variates (Edwards & Truong, 2011).

2.5 Summary

Among them the oleaginous microorganisms, the most studied microbes are yeast and algae due to their high cellular lipid contents. Microalgae, such as *Chlorella* sp., *Nannochloropsis* sp., and *Scenedesmus* sp. have been reported as promising candidates for biofuel productions as a result of their remarkably high lipid productivity, and rapid growth compared to other energy crops (Mata et al., 2010). Although photoautotrophic microalgae grow much faster than terrestrial crops, they grow much more slowly than many heterotrophic oleaginous microorganisms such as bacteria and yeast (Dong et al., 2016). Moreover, lipid content in microalgae can vary widely depending on the species (Liang et al., 2009; Mata et al., 2010). Yeasts are regarded as advantageous than molds and algae because of their faster growth rates and ability to be cultivated easily in large scales on a wide variety of substrates (Arous et al., 2016). Several oleaginous yeasts such as *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon,* and *Lipomyces* have been extensively studied for lipid accumulation (Angerbauer et al., 2008; Zhao et al., 2011). They can accumulate more than 20% of their dry weight as lipids using synthetic media as substrates (Kumar et al., 2020). Until now, a few species of bacteria such as *Arthrobacter* sp., *R. opacus* and *Acinetobacter calcoaceticus* has been reported to accumulate a significant amount of lipids (Dong et al., 2016; Meng et al., 2009). However, bacteria could be also advantageous for accumulating lipids over other oleaginous microorganisms due to their less complex genome, cellular compartmentation, high metabolism rate, and the ability to produce a wider range of different types of lipids compared to multicellular eukaryotes (Garay et al., 2014).

Table 2.14 A short summary of research gap.

Item	Research Gap	This Study
Bioremediation	Several co-culture inoculum such as microalgae-bacteria (Cheah et al., 2018c), microalgae-yeast (Cheirsilp et al., 2011), bacteria-bacteria (Bala et al., 2015), yeast-yeast (Theerachat et al., 2017) have been studied for bioremediation of POME. However, the yeast and bacterium co-culture in bioremediation of POME has not been reported to date.	A co-culture of yeast and bacterium
Lipid accumulation	Lipid accumulation capacity of *Bacillus cereus* and *Lipomyces starkeyi* using POME as low-cost substrate has not been explored to date. Moreover, no report was found to evaluate the effect of a yeast and bacterium co-culture in microbial lipid accumulation through bioremediation of POME.	*Bacillus cereus; Lipomyces starkeyi*; Co-culture;
Extraction technique	Numerous disruption methods including physical, chemical and enzymatic have been developed to achieve selective release of biomolecules (Günerken et al., 2015; Liu et al., 2016; Middelberg, 1995; Yusaf & Al-Juboori, 2014). Pulsed electric field (PEF) technology was used for cell disruption prior to extraction of intracellular lipids only in one study for cyanobacteria *Synechocystis* PCC 6803 as feedstock (Sheng et al., 2011). However, no study has not been reported for EP technology as a cell disruption method of yeast and bacteria to enhance lipid extraction.	Electroporation
Optimization	There are only several studies for optimizing lipid production, and most of them using the OFAT and Taguchi's approach (Subhash & Mohan, 2014) (Abdelhamid et al., 2019). Only a few studies used response surface methodology (RSM) for optimizing lipid production. However, optimization of inoculum compositions along with pH, temperature, time for maximising lipid accumulation and simultaneous COD removal in a yeast-bacteria co-culture using RSM has not been studied.	Optimization of inoculum composition, pH, temperature, and time using response surface methodology

Certain bacteria native to special environments may be more prone to produce cellular lipids; for example, *Rhodococcus opacus*, *Pseudomonas* spp., and *Bacillus subtilis* (Brigham et al., 2011). It has been reported that these bacteria species can accumulate a substantial amount of lipids, especially, TAGs (Kumar et al., 2018). However, among them, *Bacillus spp.* accumulated a lipid content of 39.8% within 48 h while cultured in cotton stalk hydrolysate as a substrate as reported by Zhang et al. (2014b).

Apart from the lipid production, the strains of *Bacillus,* especially *Bacillus cereus* (*B. cereus*) and *B. subtilis*, could significantly remediate pollutants from the wastewater, as they are capable of excreting different types of enzymes such as lipase and cellulase, which would promote waste digestion (Bala et al., 2014). Therefore, using bacteria could be more advantageous for lipid production when different complex substrates, particularly, high organic wastewaters are used as feedstocks. Furthermore, *B. cereus* can survive in the harsh environmental conditions (e.g., low pH, high temperature), especially, in POME due to their spore forming capability (Grady et al., 2011). Therefore, microbial oil production and simultaneous treatment of POME wastewater by *B. cereus* would be an attractive option, as it allows for pollutant removal from the POME wastewater with a short retention time and requires a smaller land area. Recently, the symbiotic association between microorganisms are considered advantageous for enhancing lipid accumulation and bioremediation of organic wastewater. However, the effect of a yeast and bacteria co-culture in microbial lipid accumulation through the bioremediation POME is not studied. A short summary of research gap is presented in Table 2.14. In the co-culture systems, the composition of co-culture inoculum, could have significant influence on the growth performance and lipid accumulation. Besides the inoculum compositions, experimental conditions such as pH, time and temperature strongly influence the performance of microbial lipid production. However, optimization of inoculum compositions along with the afore-mentioned parameters (i.e., pH, temperature, time) which determine the performance of lipid accumulation in a yeast-bacteria co-culture has not been studied. Based on the analysis of the current situation of POME treatment, it is obvious that there is a necessity to investigate other non-traditional ways of POME valorization. Production of the microbial lipids through the bioremediation of POME could be a beneficial solution. Therefore, a comprehensive research framework was designed for this study as shown in Figure 2.24.

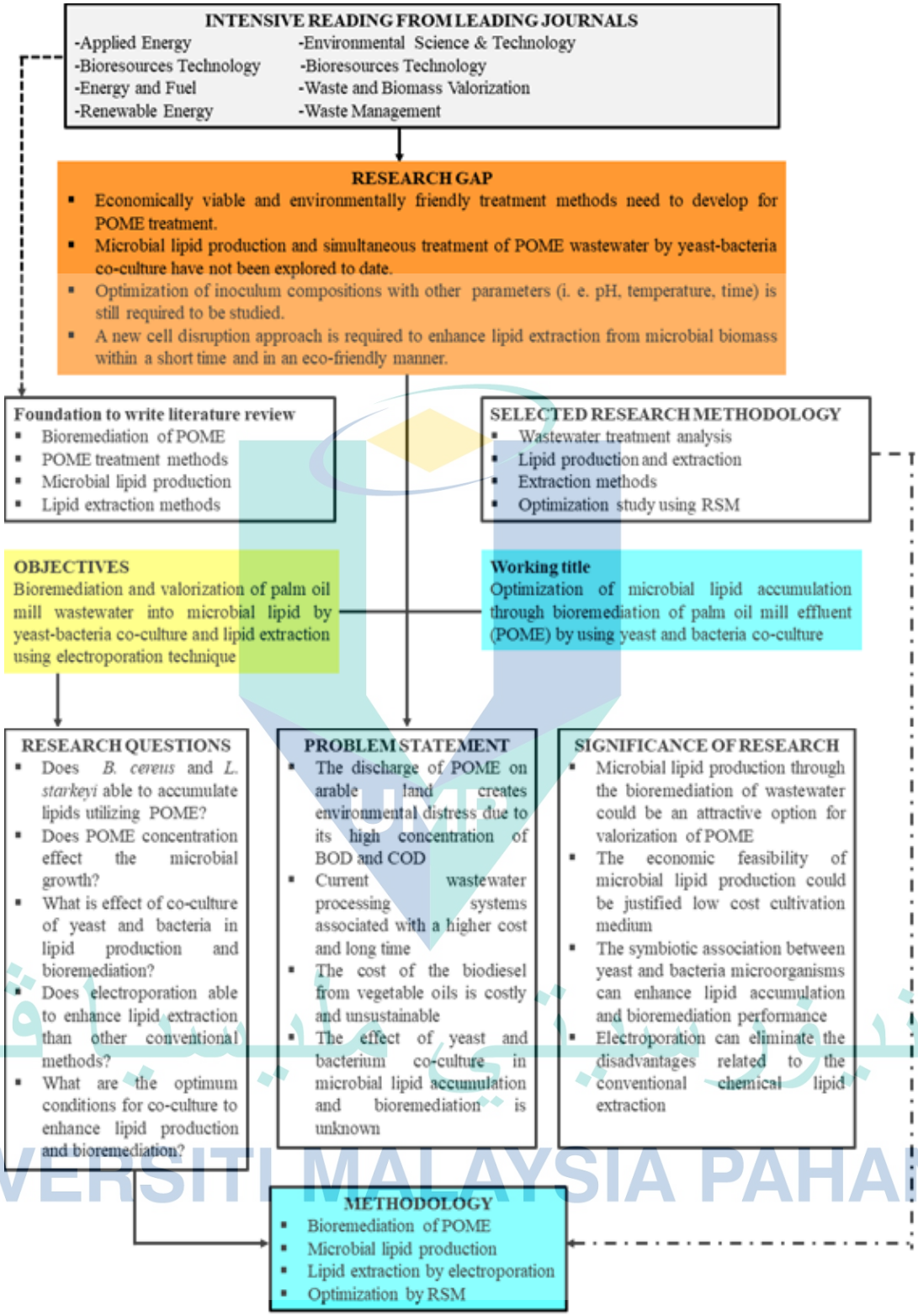

Figure 2.24 Research framework of this study.

CHAPTER 3

METHODOLOGY

The chapter described the materials and methods used in this research. The described methods include the processes for POME collection and culture medium preparation, inoculum preparation and culture conditions, POME characterization, wastewater analysis, lipid extraction process, characterization of wastewater and lipid by GC-MS, characterization of microbes by FESEM image, electroporation reactor and circuit development. Additionally, the statistical model developed by RSM for optimizing the operational parameters and maximizing the performance of the co-culture for enhanced lipid production and COD removal efficiency.

An overview of the chapter is presented in Figure 3.1. As shown from Figure, objective one focused on the bioremediation of POME with different concentrations (25, 50, 75, and 100% POME). The performance of *B. cereus*, *L. starkeyi*, and their co-culture in remediating the pollutants from POME were investigated to elucidate the efficiency of co-culture consortia of yeast-bacteria compared to monocultures. Objective 2 pointed out the efficiency of EP technique for lipid extraction compared to several conventional methods such as solvent extraction, Fenton's method, ultrasound. Objective three described the biomass harvesting, lipid accumulation capacity, and productivity and the performance of *B. cereus*, *L. starkeyi*, and their co-culture inoculum through the bioremediation of POME. Objective 4 explained the optimization of the lipid production performance and bioremediation efficiency (COD removal) using RSM to maximize the performance of the targeted co-culture inoculum. The operational parameters (inoculum composition, initial pH, incubation temperature, and time) were considered as independent variables while the COD removal efficiency and lipid production were dependent variables.

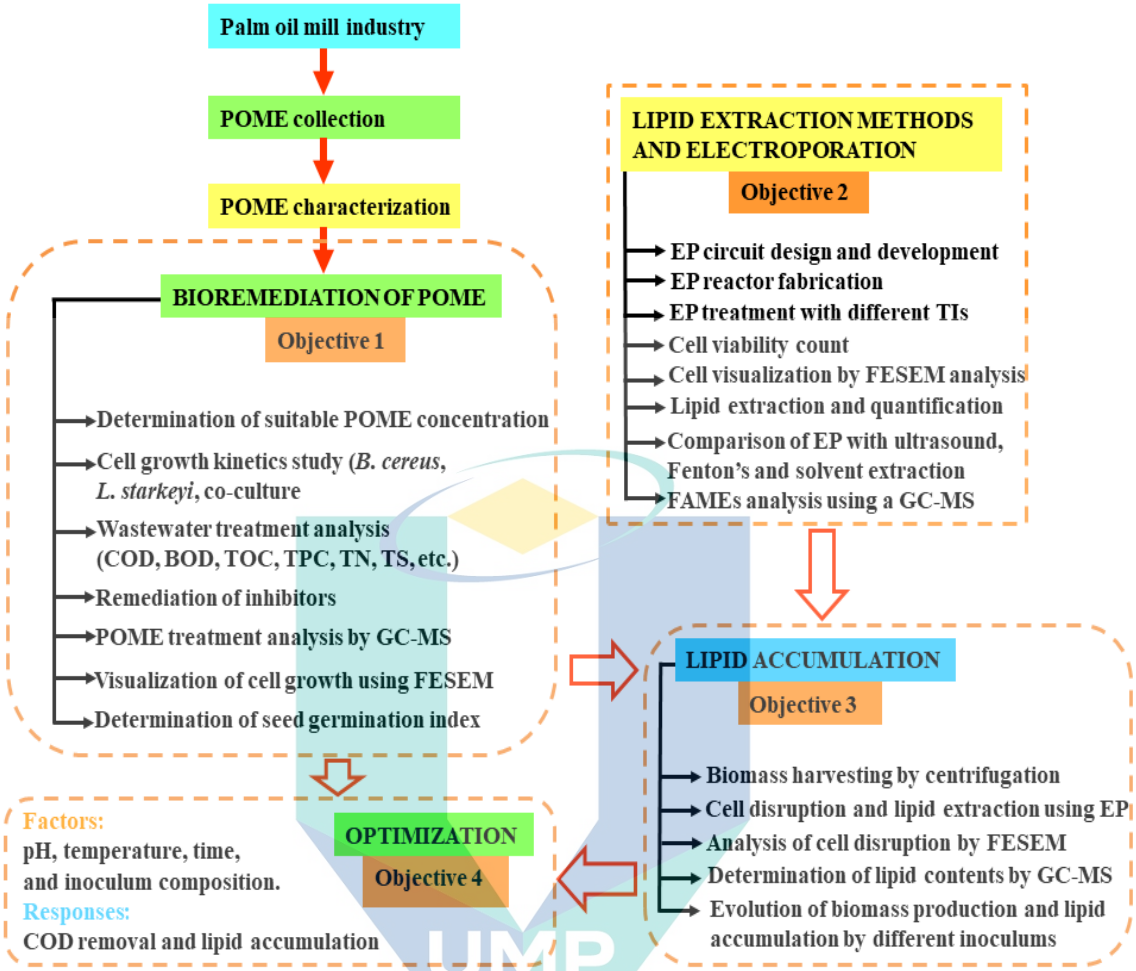

Figure 3.1 Overview of research methodology.

3.1 Bioremediation of POME using pure cultures and co-culture

3.1.1 Chemicals and reagents

The chemicals used in this study were supplied by Shanghai Sunny Scientific Chemicals. Distilled water and deionized water were obtained from the Bioprocess laboratory of University Malaysia Pahang (UMP). A complete list of the chemicals and materials used in this study is presented in Table 3.1

Table 3.1 List of chemicals and materials.

Chemical and Materials	Source	Purity
$KMnO_4$	Sigma-Aldrich	99%
Hydrochloric acid (HCl)	Sigma-Aldrich	60%
Sulphuric acid (H_2SO4)	Sigma-Aldrich	99%
Ethanol	Sigma-Aldrich	70%
Phosphate buffer	Sigma-Aldrich	-
Calcium chloride	Sigma-Aldrich	-
Ferric chloride solution	Sigma-Aldrich	-
Filter disk	Sigma-Aldrich	-
Weighing dish	Sigma-Aldrich	-
Petriplates	Sigma-Aldrich	-
Glutaraldehyde	Sigma-Aldrich	25%
Formaldehyde	Sigma-Aldrich	1%
$MgCl_2$	Sigma-Aldrich	-
n-hexane	Sigma-Aldrich	Pure grade
KH_2PO_4	Sigma-Aldrich	-
$MgSO_4.7H_2O$	Sigma-Aldrich	-
$(NH_4)_2SO_4$	Sigma-Aldrich	-
Yeast Extract	Sigma-Aldrich	-
Peptone	Sigma-Aldrich	-
Glucose	Sigma-Aldrich	-

3.1.2 POME collection and culture medium preparation

Raw wastewater was collected from a local palm oil mill (LKPP Corporation Sdn. Bhd.) located in Gambang (Longitude: 103.102686, Latitude: 3.709472), Pahang, Malaysia, before the effluent was discharged into the fermentation pond. The samples were stored in sterilized glass bottles at 4 °C to avoid the deterioration of the organics in the POME. The submerged solids and debris present in the raw POME were removed using a Whatman No. 1 filter paper. The undiluted raw POME (filtrated by Whatman No. 1 filter paper) was considered as the 100% POME sample, and the remaining 75, 50, and 25% POME samples were prepared by diluting the raw POME as described in Table 3.2. The pH was adjusted to 7.0 ± 0.1 by adding 1N NaOH for all samples.

Table 3.2 Definition of POME samples.

Sample	Definition
100% POME	Undiluted (raw POME), after filtration to remove solid particles
75% POME	Raw POME and de-ionized water, 3:1
50% POME	Raw POME and de-ionized water, 1:1
25% POME	Raw POME and de-ionized water, 1:3

3.1.3 Inoculum preparation and culture conditions

In this study, a wild type pure culture of *B. cereus* and ATCC culture of *L. starkeyi* were used as inoculum. The strain of *B. cereus* (accession no. MF 661883) (Islam et al., 2016) was attained from the Laboratory of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, Malaysia. Whereas, the pure culture of *L. starkeyi* ATCC 56304 was obtained from the, University of Naples Federico II (Laboratory of Biochemical Engineering), Italy. The 10 mL agar slants (agar, 2% w/v; yeast extract, 10 g/L; peptone, 10 g/L and glucose, 10 g/L) were prepared to grow and maintain the stock culture. The liquid culture of bacteria was prepared in Luria-Bertani broth (10% v/v) as growth medium at 35 ℃ and 150 rpm overnight. Thereafter, the fresh bacterial cells were prepared by sub-culturing 1 mL of broth on the solid agar Petri plates. Finally, the primary inoculums of both bacteria and yeast were prepared in 10 mL of sterile water by inoculating 10 loops from the solid Petri plates and slants, respectively. The ratio of *B. cereus* and *L. starkeyi* was maintained as 1:1 to prepare co-culture inoculum. The initial cell concentrations of all inoculums were adjusted using a UV spectrophotometer (Shimadzu UV, model: UV-180 240 V) at OD_{600}=1.5 for the subsequent experiments.

3.1.4 Determination of cell concentration and growth kinetics

The survivability and growth kinetics of *B. cereus* and *L. starkeyi* in different POME concentrations (25, 50, 75, and 100%) were studied for 6 days (144 h). In brief, the 200 mL of POME was put into Erlenmeyer flasks (500 mL) and sterilized at 121 °C for 20 min followed by a reduction to ambient temperature. The 1 mL of primary inoculum (*B. cereus* and *L. starkeyi*) was then inoculated into the different POME samples and incubated at 35 ℃ (bacteria)/25 ℃ (yeast)and 150 rpm for 6 days. A batch

was run using a synthetic medium (D-glucose, 1 g/L; peptone, 15 g/L; sodium chloride, 6 g/L; yeast extract, 3 g/L) as a control. In the case of coculture inoculum, the 50% POME samples were inoculated by the 1 mL of primary inoculum (*B. cereus*, *L. starkeyi*, and co-culture consortium) and incubated at 30 ℃ with 150 rpm for 144 h. The entire experiment was conducted with only POME and without adding any commercial nutrients. The 1 mL POME samples (serially diluted up to 10^{-6} CFU/mL) were spread on nutrient agar Petri plates in 12 h intervals during the 6 days of enrichment. Finally, the colony forming units (CFU) were calculated after 24 h to evaluate the growth kinetics of *B. cereus*, *L. starkeyi*, and co-culture consortium.

3.1.5 Wastewater treatment analysis

The organic contents present in POME (BOD, COD, TOC, TPC, TSS, TDS, TS, TN, AN, NN, oil and grease etc.) were determined before treatment and after treatment by following APHA methods (Federation & Association, 2005). The removal efficiency of different parameters was calculated by using Equation 3.1.

$$Removal\ efficiency\ (\%) = \frac{(C_i - C_f)}{C_i} \times 100 \qquad 3.1$$

where C_i is the initial concentration of pollutants in POME before treatment and C_f is the final concentration of those contents after 6 days of treatment. All tests were conducted three times to check the reproducibility.

3.1.5.1 Chemical oxygen demand measurement

A measured volume (1 mL) of raw POME was mixed with 49 mL of distilled water in a beaker and stirred to mix. The mixed solution (2 mL) was added into a vial containing a digestion solution made of high-range COD reagent (20-1500 mg/L), while a control solution was set up by adding 2 mL of distilled water into a digestive solution. Later, the vials were capped and mixed by inverting the vial twice. Both solutions were heated using a thermo reactor (DRB-200, Hach USA) at 150 ℃ for 2 h and later cooled to room temperature. The solution prepared with distilled water was used to zero the spectrophotometer (DR-2800, Hach), while the amount of COD in the POME solution

was determined spectrophotometrically. The amounts of COD in other diluted samples were similarly determined.

3.1.5.2 Biochemical oxygen demand measurement

One liter of diluted water sample was prepared by adding 1 mL of phosphate buffer, magnesium sulphate, calcium chloride, and ferric chloride solution into a 1 L volumetric flask before making up the final volume to 1 L using distilled water. Later, 10 mL of POME was added into a 500-mL beaker and made up to 300 mL using the prepared dilution water. A further 300 mL of dilution water was prepared and used as a control solution. All the prepared samples and control were each put into 300 mL-incubation bottles. The dissolved oxygen (DO) concentration of the preparations was measured and recorded using a DO meter. A small quantity of water was added into the flared mouth of the bottle before covering the bottles with aluminum foil. The bottles were placed in a BOD_5 incubator and left for five days at 20 °C. The final DO value was measured, while the BOD value was calculated using Equation 3.2.

$$BOD\ \left(\frac{mg}{L}\right) = DO_i - DO_f \times dilution\ factor \qquad 3.2$$

The dilution factor is determined by dividing the bottle volume with the sample volume, while DO_i is the initial value and DO_f is the final value of DO.

3.1.5.3 Total solids

A measured volume (5mL) of the well-mixed sample was transferred to a pre-weighed dish under a continuous magnetic stirring. Subsequently, the sample was evaporated in a drying oven at a temperature of approximately 2 °C below boiling point to prevent splattering. The evaporated sample was later dried for at least 1 h in an oven at 103 to 105 °C before being transferred to a desiccator for temperature balancing and weighing. The cycle of drying, cooling, desiccating, and weighing was repeated until a constant weight was obtained. At least 10 % of all samples were analyzed in duplicate, and the duplicated determinations agreed within 5% of their average weight. The total solid was measured using Equation 3.3.

$$mg\ of\ total\ solids/L = \frac{(A - B) \times 1000}{sample\ volume, mL} \quad 3.3$$

where A is the weight of the dried residue and dish in mg, and B is the weight of the dish in mg.

3.1.5.4 Total dissolved solids

Initially, the sample was stirred with a magnetic stirrer and a measured volume was pipetted into a glass-fiber filter under vacuum. Subsequently, the sample was washed with three successive changes of 10 mL reagent-grade water, allowing a completed drainage between washings, with continued suction for about 3 min after filtration. The total filtrate (with washings) was later transferred to a pre-weighed evaporating dish and evaporated to dryness in a drying oven. Thereafter, the evaporated sample was dried for at least 1 h in an oven at 180 ± 2 °C and cooled in a desiccator before weighing. The cycle of drying, cooling, desiccating, and weighing was repeated until a constant weight was obtained. At least 10% of all the samples were analyzed in duplicate and the duplicated determinations agreed within 5% of their average weight. The total dissolved solid was measured using Equation 3.4.

$$mg\ of\ total\ dissovled\ solids/L = \frac{(A - B) \times 1000}{sample\ volume, mL} \quad 3.4$$

where A is the weight of the dried residue and dish in mg, and B is the weight of the dish in mg.

3.1.5.5 Total suspended solids

Initially, the disk was inserted in a filtration apparatus with the wrinkled side facing up. Thereafter, the disk was washed with three successive changes of 20 mL reagent-grade water under vacuum. The suction process was sustained until all the traces of water were removed. Then, the vacuum was turned off, and the washings discarded. Then filter was removed from the filtration apparatus and transferred to an inert aluminum weighing dish. The filter was dried in an oven at 103 to 105 °C for 1h before being cooled in a desiccator prior to weighing. The repeated cycle of drying or igniting, cooling, desiccating, and weighing was repeated until a constant weight was obtained.

After achieving a constant weight, the sample was stored in a desiccator until needed. At least 10% of all the samples were analyzed in duplicate and the duplicated determinations agreed within 5% of their average weight. The total solid was measured using Equation 3.5.

$$mg\ of\ total\ suspended\ solids/L = \frac{(A - B) \times 1000}{sample\ volume, mL} \qquad 3.5$$

where A is the weight of the dried residue and the dish in mg, and B is the weight of the dish in mg.

3.1.5.6 Ammoniacal nitrogen

About 0.1 mL of the sample was added to Am Ver TM diluent reagent test N tube for H high range ammonia nitrogen and made up to 5 mL using deionized water. A blank was also prepared by adding 5 mL deionized water to Am Ver TM Diluent Reagent Test Tube for High range ammonia nitrogen. Subsequently, ammonia salicylate reagent powder pillow was added into the sample and blank vials and allowed for 3 minutes. After 3 minutes, ammonia cyanurate reagent powder was added into both vials and allowed for another 15 minutes. After the incubation period, the blank vial was used to zero the spectrophotometer (HACH spectrophotometer DR5000) before reading the concentration of ammonia in the sample. The concentration of NH_3-N in the sample was displayed in mg/L.

3.1.5.7 Total phenolic content

The total phenolic content was determined using the spectrophotometric method (Singleton & Rossi, 1965). A methanolic solution of the extract at a concentration of 1 mg/mL was used in the analysis. The determinations were carried out using the Folin–Ciocalteu reagent. The results were expressed as GAE (Gallic Acid Equivalents).

3.1.6 Remediation of inhibitors

To observe the bioremediation of POME by *B. cereus*, *L. starkeyi* the removal of the major pollutants (BOD, COD, TOC, TPC) were studied at 12 h intervals during 144

h for 50% POME. The COD removal efficiency was then compared with co-culture inoculum using 50% POME. All tests were performed by following APHA methods as described above.

3.1.7 POME treatment analysis by gas chromatography–mass spectrometry

To observe the pollutants removal from POME, the organic compounds in the 50% POME were characterized before treatment and after treatment by *B. cereus*, *L. starkeyi*, and co-culture consortium. In brief, the organic compounds were extracted from POME using liquid–liquid extraction with n-hexane. Fixed volumes (1 µL) of extracted samples were analyzed using Gas Chromatography–Mass Spectrometry (GC-MS, Agilent Corporation, USA). Extremely pure He (99.99%) was used as the carrier gas at a flow rate of 1 mL/min. A DB-35MS capillary column with an inner diameter of 0.25 mm and a length of 30 m was used in the separation system. The temperature of the gasification compartment was set to 40 °C for 5 min and then increased to 280°C at a rate of 3°C /min.

3.1.8 Visualization of cell growth using field emission scanning electron microscopy

The cell growth was visualized under field emission scanning electron microscopy (FESEM, JEOL JSM-7800F, Japan). The samples of the bacteria, yeast, and co-culture cells were collected from the reactor and placed on a glass slide, then dried at room temperature (27 ± 2 °C) for 2 h. Subsequently, all samples were coated with platinum using an ion-sputtering technique. Finally, the cells were visualized under the microscope.

3.1.9 Determination of seed germination index

The seed germination test was performed using the seeds of mung bean (*Vigna radiata*), purchased from the Tunas mart located at Gambang, Pahang, Malaysia. Prior to germination, the seeds were treated for 2 min with 0.2 N mercuric chloride and washed with distilled water to remove contaminations on the seed coats (Koutinas et al., 2014). The fixed volume of untreated and treated 50% POME (by *B. cereus*, *L. starkeyi*, and co-culture consortium) were put on sterilized Petri plates. The seeds placed in a Petri plate containing distilled water were considered as a control, while untreated raw POME

(100% POME) were considered as a reference. Twenty seeds were germinated in each Petri plate at room temperature (27 ± 2 °C). All experiments were repeated three times to confirm the observations of each treatment. The number of germinated seeds were counted at 12 h intervals for 3 days. The germination index (GI) was evaluated using Equation (3.6).

$$GI = \frac{N_i}{S} \times T_i \qquad 3.6$$

where T_i = duration of planting (days); N_i = amount of germinated seeds on day i (quantity); and S = total planted seeds (quantity).

3.2 The efficiency of electroporation technique in microbial lipid extraction

3.2.1 Electroporation circuit design and development

The Arduino programming language (based on Wiring) was written on the Arduino Software (IDE) to control the circuit. Pulse-width modulation (PWM) processing was used to control the pulses provided by the generator, which produced high voltage (4 kV) with 2-5 A current depending on the load. The power supply of this circuit was 6 V. A lithium battery of 6 V with 12 Ah capacity was used to ensure a high performance for the circuit. Transistor 2N222 was used to transfer PWM signal from Arduino to the circuit, which reduced the noise that might disturb the main circuit. The 470 Ω resistor was loaded to decrease the voltage and limit current before it entered the base of transistor as well as to protect the transistor. However, the Arduino circuit controlled the range of frequency from 0 Hz to 100 Hz with the increment of 10 Hz. The frequency was measured by time domain of Arduino using the formula, f=1/T. The designed circuit was used for controlling the frequency to get square wave output. The high voltage pulses were calculated as 0.05 ms until 10 ms with 4 kV. The voltage and total current were monitored by using an oscilloscope. The single pole double throw relay was used to act as a control switch in the controller circuit for connecting and disconnecting the pulses. The whole set-up including the reactor and pulse generator is presented in Figure 3.2.

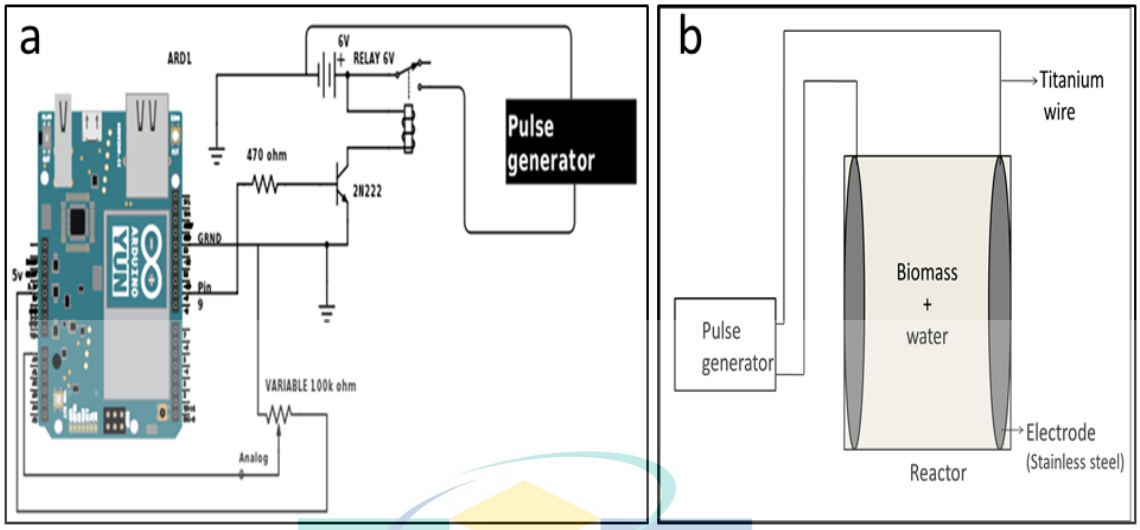

Figure 3.2 Schematic diagram of (a) pulse generator circuit and (b) electroporation reactor.

3.2.2 Electroporation reactor fabrication

Three EP treatment devices (R_1, R_2, and R_3) were designed and fabricated using plexiglass which had dimension of 5 cm x 7.5 cm x 5 cm, 5 cm x 5 cm x 5 cm, and 5 cm x 2.5 cm x 5 cm, respectively. The total working volume of R_1 was 30 mL whereas that of R_2 and R_3 were 20 mL and 10 mL, respectively. Two circular stainless-steel plates with a surface area of π $(1.8)^2$=10.17 cm^2 were used as electrode in all devices. The distances between electrodes were fixed at 6 cm, 4 cm, and 2 cm for R_1, R_2, and R_3, respectively.

3.2.3 Cell culture and biomass collection

The oleaginous yeast strain *L. starkeyi* (ATCC 56304) was collected from the laboratory of Biochemical Engineering, University of Naples Federico II, Italy. The strain was cultured in YPD agar (m/v: 1% yeast extract, 2% peptone, 2% dextrose/glucose, 2% agar) slants to maintain a stock culture. The subculture was done on petri plates in order to grow new cells. The primary inoculum was prepared by dissolving 10 loops of microbes from the sub-cultured petri plate in 10 mL of sterilized water. The 150 mL of synthetic medium was taken in 500 mL Erlenmeyer flasks and sterilized at 121 °C for 20 min. The medium was prepared by dissolving 1.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 2.0 g $(NH_4)_2SO_4$, 0.5 g Yeast Extract, 2.0 g Peptone, and 70.0 g of Glucose in 1L DI water (Yousuf et al., 2010). Thereafter, the medium was inoculated with 1 mL of primary

inoculum and incubated at 25 °C for 4 days with a rotation speed of 150 rpm. The biomass was separated using centrifugation technique (at 10000 rpm for 10 min). The weight of collected biomass was measured by analytical balance. In case of dry biomass, the wet biomass was oven dried at 40 °C until getting constant weight.

3.2.4 Electroporation treatment

The 1.2 g of wet biomass was mixed with 60 mL of sterile water. The 30 mL, 20 mL and 10 mL of mixtures were taken in the EP reactor R_1, R_2 and R_3 respectively. The cell concentrations were adjusted by measuring the optical density at 600 nm (Shimadzu model UV-160A) for each reactor to maintain equal concentration of cell at initial. The initial cell concentration was approximately 0.9 x 10^5 CFU/mL. Sample conductivity was 5.5 x 10^{-6} S/m and the initial temperature was maintained at (27 ± 2 °C). The high voltage (4 kV), with the frequency of 100 Hz was employed to the reactor. All experiments were triplicated to confirm the observation of each treatment.

3.2.5 Experimental conditions

The EP device was operated through a range of controllable operating variables (voltage, frequency, distance between electrodes, treatment time etc.) to understand the factors that increase the amount of cell inactivation. To integrate all of these variables, treatment intensity (TI) (Salerno et al., 2009) was calculated using the following Equation 3.7:

$$TI = K\frac{V^2 . D . f . \sigma . HRT}{L2} \qquad 3.7$$

Where TI is the treatment intensity in kWh/m^3, V is the applied voltage in kgm^2/C s^2, D is the pulse width in s/pulse, f is the pulse frequency in pulse/s, σ is the sample conductivity in S/m (= s C^2/ kg m^3), L is the distance between electrodes in m, HRT is the hydraulic residence time in the treatment chamber in s, and K is a constant for unit conversion, 2.778 x 10^{-7} kWh/J.

3.2.6 Effect of electroporation on cell viability

To evaluate the viability of cells under EP treatment and control conditions, spread plate technique was followed. The 20 µL of sample was taken from reactor before and after EP treatment of 2, 4, 6, 8 and 10 min and mixed with 980 µL sterile water. Each sample (20 µL + 980 µL = 1 mL) was then spread onto the petri-plates (YPD agar media) and incubated at 25 °C for 48 h. After that, the CFU were counted to calculate cell inactivation rate.

3.2.7 Field emission scanning electron microscopy analysis

The disruption of yeast cells was observed using FESEM (JEOL JSM-7800F, Japan). The samples were collected from EP reactors before and after treatment and taken over selective slides. The samples were then dried with a critical-point dryer and coated with platinum (using an ion-sputter) to a thickness of 10 nm. Finally, the cell wall breakup was visualized in FESEM image.

3.2.8 Lipid extraction and quantification

To extract microbial lipid, the treated biomass with water was collected in a falcon tube at the end of the electroporation process and followed by mixing with 10 mL solvent (chloroform: methanol= 2:1). Then the biomass was separated, and lipid-rich supernatant was isolated by centrifugation (at 4000 rpm, 10 min). Thereafter, the biomass was washed 2 times with 5 mL solvent and vortexed properly followed by centrifugation for 10 minutes (at 4000 rpm) to separate solid residuals from the supernatant liquid where lipids were transferred. All liquid portions were stored in a tube and after centrifugation; the lower liquid (organic) phase was withdrawn by Pasteur pipette and taken into a watch glass. The liquid was evaporated using an oven drier at 40 °C until getting a constant weight and calculated the extracted lipid. To compare the lipid extraction performance of EP with other techniques, another batch (1st batch) of EP was conducted for 10 min in reactor R_3. The 50 mg of dry biomass (*L. starkeyi*) was mixed with 10 mL of sterile water and EP treatment was followed as stated before in EP treatment section. In 2nd batch, in absence of EP, the 10 mL of solvent (chloroform: methanol= 2:1) was added in the same amount (50 mg) of dry biomass and shaken (10 min) for mixing properly. In 3rd batch, Fenton's reagent (500 µL of $FeSO_4$ and H_2O_2 1.5:10 solution) was added in the equal

amount (50 mg) of dry biomass with same amount (10 mL) and similar standard of methanol and chloroform to disrupt the cell wall. In 4th batch, the chloroform extraction was conducted in the ultrasonic bath at room temperature (27 ± 2 °C), for 10 min, at a frequency of 35 kHz with a power of 240 W. The 10 mL of solvent with same standard, 50 mg of biomass and treatment time for 10 min were maintained for all above-mentioned batches. The lipid extraction and recovery method were followed as described above. Finally, the percent weight of lipids extracted from the dry biomass was obtained as the ratio between the weight of lipid obtained and the original dry weight of yeast biomass which was subjected to the extraction process. All experiments were triplicated to confirm the observation of each treatment.

3.2.9 Lipid characterization

To study the lipid composition, a separate batch was prepared by employing the EP treatment for the direct transesterification process. A solvent of methanol-chloroform (ratio 2:1) was added to the biomass at a solvent-biomass ratio of 10:1 (mL:g) to extract and transesterify the lipids in the presence of H_2SO_4 (0.75%). The reaction was carried out at room temperature for 100 h (Thanh et al., 2012). To separate fatty acid methyl esters (FAMEs), 4 mL of n-hexane was added to the sample after the completion of transesterification. The mixture was thoroughly vortexed for 30 s and centrifuged to disperse the layers. The hexane layer containing FAMEs was immediately analyzed using GC-MS. A DB-35 MS capillary column (inner diameter 0.25 mm and a length of 30 m) was used in the separation system. Highly pure (99.99%) helium (He) was used as a carrier gas with a flow rate of 1.0 mL/min. The inlet temperature was held at 225 °C. The temperature of the gasification compartment was set to 100 °C (held for 1.0 min) and amplified by 10 °C/min up to 250 °C (held for 5.0 min).

3.3 Lipid accumulation by *B. cereus*, *L. starkeyi* and their co-culture through bioremediation of POME

3.3.1 Biomass harvesting methods

The microbial biomass was collected from 200 mL of liquid cultures after the centrifugation (10,000 rpm for 10 min) technique and weighed using an analytical balance. Briefly, *B. cereus* and *L. starkeyi* (primary inoculum) was inoculated at different

concentrations of POME (25, 50, 75, and 100%) and the cultures were enriched for 6 days. Thereafter, the biomass was separated using the centrifugation technique (at 10,000 rpm for 10 min) and weighed using the analytical balance. The dry biomass was obtained by drying the wet biomass in an oven at 40 °C until a constant weight was measured. Likewise, subsequent batches of *R. opacus* ATCC 51881 and *P. aeruginosa* ATCC 15442 were conducted in 50% POME to compare the biomass and lipid accumulation capacity of *B. cereus* with typical bacterial strains. In co-culture inoculum, the primary inoculum *B. cereus* and *L. starkeyi* was mixed with a ratio of 1:1 and inoculated at different concentrations of 50% POME and the cultures were enriched for 6 days and collected by following similar procedure as described above.

3.3.2 Cell disruption and lipid extraction

The EP technique was applied to disrupt the cell wall to facilitate the lipid extraction process, according to the section 3.2.4. In brief, 50 mg of dry biomass was placed in the reactor, and EP treatment was performed by applying the electrical pulses (4 kV) with a frequency of 100 Hz for 10 min. Finally, the percent weight of lipid extracted from the biomass was calculated as the ratio of the weight of lipid achieved and the actual dry weight of biomass subjected to the extraction process. All the experiments were replicated three times. The lipid content and lipid productivity were then calculated with Equation (3.8) and Equation (3.9), respectively.

$$Lipid\ content\ (\%) = \frac{W_2}{W_1} \times 100 \qquad 3.8$$

where W_1 = total biomass in dry weight basis (mg/L) and W_2 = total lipid content (mg/L).

$$Y = \frac{L \times W_1}{t} \qquad 3.9$$

where Y = lipid productivity (mg/L/day); L = lipid content (%); W_1 = total biomass content (mg/L); and t = total duration of cultivation (days).

3.3.3 Analysis of cell disruption

The effect of the EP technique on cell disruption of bacteria and yeast cell was visualized by using FESEM (JEOL, JSM-7800F, Japan) operated at a voltage of 7 kV. The samples of the cell were collected from the EP reactor before and after the treatment and placed on a glass slide. The samples were then dried at room temperature (27 ± 2 °C) for 2 h. Subsequently, all samples were coated with platinum using an ion-sputtering technique. Finally, the cells were visualized under the microscope.

3.3.4 Determination of lipid contents

To analyze the lipid content and composition accumulated by *B. cereus* and *L. starkeyi*, a separate batch was conducted with wet biomass (400 mg) in the transesterification vial, avoiding the biomass drying and lipid extraction stages. Thereafter, the transesterification reaction and FAMEs analysis via GC-MS were carried out following the method as described in the section 3.2.9.

3.3.5 Evolution of biomass production and lipid accumulation

To study the kinetics of biomass growth and lipid accumulation of *B. cereus*, *L. starkeyi* and their co-culture were studied at 12 h intervals in the 50% POME during 144 h of treatment. The microbial biomass was harvested after 6 days of enrichment. Briefly, the biomass was separated from microbes enriched liquid cultures (200 mL) by centrifuging (10,000 rpm for 10 min) and weighted by an analytical balance. To obtain dry biomass, the wet biomass was dried in an oven at 40 ℃ until a constant weight was observed. Thereafter, the microbial lipids were extracted by using EP technique. Briefly, the dry biomass (50 mg) was taken in the EP reactor, and thereafter 4 kV of electrical pulses was applied for 10 min with a frequency of 100 Hz. The treated mixture was centrifuged for 10 min (at 5000 rpm) to separate the solvent phase. Finally, a Rotavapor (Buchi, R-100) was used to obtain dry lipids.

3.4 Optimization of yeast-bacteria co-culture

3.4.1 Response surface methodology analysis

Response surface methodology (RSM) is a statistical modelling approach for determining the relationship between various process parameters and the responses, and to determine the significance of the influence of these process parameters on the response (Thakur et al., 2009). The central composite statistical design (CCD) was used to study the interactive effects of the independent variables. This design is used for fitting the second-order polynomial model to the experimental runs. The levels in 2^k design are denoted as low, intermediate, and high with the assumption of 0 for low, 1 for intermediate and 2 for high, given that k represents the number of factors included in the study. Here, the two-level statistical design of RSM was performed for lipid accumulation (mg/L) and COD removal efficiency (%) as the dependent variable (response). The RSM model using CCD factorial design suggested a total of 30 experimental runs. The range and levels of the processing parameters involved in this design are presented in Table 3.3. In this study, the independent variables were inoculum compositions, pH, temperature and time, while the dependent parameters were COD removal efficiency and lipid accumulation.

Table 3.3 Variables for optimizing the performance of co-culture inoculated reactor.

Name	Units	Type	Low	High
Concentration of microorganism A in inoculum*	Vol %	Factor	30	70
pH	-	Factor	6	7
Time	h	Factor	80	100
Temperature	℃	Factor	30	35
COD removal efficiency	%	Response	73	82
Lipid accumulation	mg/L	Response	1.67	1.58

* A- *B. cereus* in co-culture inoculum, where B is *L. starkeyi*

3.4.2 Experimental design and data analysis for optimization

The COD removal efficiency and lipid accumulation capacity were considered as dependent variables, whereas the inoculum composition, medium pH, incubation temperature, and cultivation time were regarded as predictor variables to establish a mathematical model. The approach of sequential analysis using design of experiments was proposed where each regressor was coded as follows (Equation 3.10).

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \left(\sum_{i=1}^{n} b_{ii} x_i\right)^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} b_{ij} x_i x_j \qquad 3.10$$

where, Y= predicted response; b_i=linear coefficient; b_0=constant coefficient; b_{ij}=interaction of coefficient; and x_i, x_j are coded values of a reactor.

Generally, the main objective of RSM is to optimize the response (Y) based on the considered factors (Zularisam et al., 2007). The Design Expert software (Version-7.1.6) was used to develop the experimental design and optimize the regression equation (Equation 3.10). The statistical significance of the model equation was determined by performing Fisher's statistical test for analysis of variance (ANOVA). A good model must be significant based on the F-value and P-value as opposed to the Lack-of-Fit (insignificant). Furthermore, the proportion of variance exhibited by the multiple coefficients of determination R^2 should be close to 1 as this value signifies a better correlation between the experimental and the predicted values. In more detail, P-value is the probability of the null hypothesis to occur. The 'P' in P-value stands for 'Probability'. The null hypothesis in ANOVA is 'Assuming that the means of the data groups of each factor are equal'. Generally, if this percentage is 5% or less, you can reject a null hypothesis. ANOVA F-value used for feature selection. If the features are categorical, calculate a chi-square (x^2) statistic between each feature and the target vector. However, if the features are quantitative, compute the ANOVA F-value between each feature and the target vector. The F-value scores examine if, when we group the numerical feature by the target vector, the means for each group are significantly different.

3.4.3 Statistical analysis

The results obtained from experimental run were analyzed by Design Expert (Version-7.1.6). The model adequacy and error independency were used for each of the variables to diagnose the fitted models. The significance of the fitted model was evaluated by using the ANOVA. Furthermore, different statistics were used to analyze the adequacy of the model and the coefficient of determination, R^2 was used to assess the integrity of the fitted model. In addition, the ratio of the signal to noise was evaluated by adequate precision statistics (Madani et al., 2015; Mishra et al., 2019). How well the model fitted with the i^{th} point and how far that point was from the rest of the data were evaluated by using Cook's distance statistics. The large distance data (greater than unity) requires to be assessed with more attention since the point is more influential than the other (Madani et al., 2015). Normal probability plot of the residuals was used to check the normality of the errors. The consistency of variances was measured by plotting the residuals versus the time sequence of the runs, predicted values, and each independent variable. The data transformation on the response was used to ease the issues, while the discrepancies were observed. The replicates of center points were inserted to the factorial designs to analyze the adequacy of the model to capture the curvature expressed in the response. The set of equations derived from the differentiation of the fitted model was solved to calculate the predicted response for optimum value and their levels of independent variable.

CHAPTER 4

BIOREMEDIATION EFFICIENCY OF POME BY *B. CEREUS, L. STARKEYI* AND THEIR CO-CULTURE

4.1 Introduction

In this chapter, an approach has been presented to bioremediate POME using a pure culture of *B. cereus*, *L. starkeyi*, and their co-culture inoculum. The POME concentration was optimized for enabling a maximum yield of biomass growth. The bioremediation efficiency of different inoculums was investigated and characterized by pollutant removal efficiencies, FESEM, GC-MS. The degree of bioremediation was further justified by determining seed germination indices.

4.2 Biomass growth profile of *B. cereus*

The growth kinetics of *B. cereus* were studied using different concentrations (25, 50, 75, and 100%) of POME as presented in Figure 4.1. As seen from Figure 4.1, the cell growth of *B. cereus* followed a similar trend for all concentrations of POME, including the synthetic medium (control) even though a differential stationary phase was observed for different concentrations. However, among the four concentrations, the maximum growth was achieved for 50% POME, although the initial growth rate was slightly higher for 25% POME. Additionally, the cell growth took a higher retention time to reach a plateau for the lower dilution (75% POME).

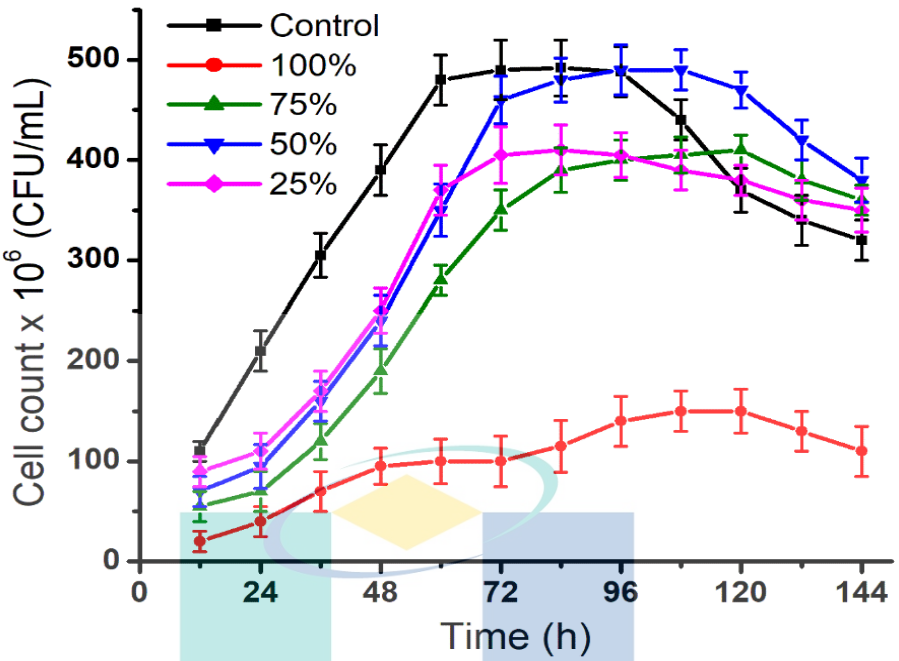

Figure 4.1 The growth profile of *B. cereus* in the POME.

The 100% POME sample obtained a lower cell growth than the other POME samples. This lower cell growth might be attributed to the fact that this condition was not favourable for the assimilation of *B. cereus* growth, although there were more carbon and nutrients present in the raw POME. This was probably due to the presence of a higher load of organics, phenolic compounds, and long chain fatty acids, which might act as inhibiting agents. Consequently, these agents hindered the metabolic power and growth of microorganisms (Cheah et al., 2018d; Islam et al., 2018c). These compounds are known to be present in POME and have been reported to have both antibiotic and phytotoxic properties (Uzel et al., 2005). In addition, the higher concentration of total nitrogen in raw POME (Table A1, in appendix) might have inhibitory effects on growth kinetics (Cheah et al., 2018a), as *B. cereus* grows in a nitrogen limiting medium. However, the dilution of the samples has been shown to decrease the impact of these compounds, allowing the microbes to flourish and metabolize the organic materials present in the effluent. Therefore, the higher growth rate was observed corresponding to a diluted POME medium (75%, 50% and 25% POME). This may be explained by the lowering of nutrient concentration due to the higher dilution (25% POME) because the nutrients present in the 25% POME were quickly utilized and thereby the growth maxima could not be reached, as in the 50% POME. Nevertheless, the highest growth observed in the 50% POME sample might be accredited to the favorable compositions (i.e., lower

inhibitors, suitable carbon/nitrogen ratio, etc.) of the nutrients in this broth (Yousuf et al., 2010). The findings obtained in the present study were in good agreement with those previously reported for yeast and microalgae (Cheah et al., 2018b; Islam et al., 2018c).

4.3 POME characterization and bioremediation efficiency of *B. cereus*

The 50% POME (the optimal concentration, as shown in the section 4.2) sample was characterized by analyzing BOD, COD, TOC, TPC, AN, NN, TN, TSS, TS, and TDS before and after treatment by *B. cereus*. After being treated with *B. cereus*, a significant reduction was observed for each parameter, as presented in Table 4.1. The substantial reduction in organic load, particularly in BOD (72.65%) and COD (79.35%) was observed after 144 h of treatment.

Table 4.1 Bioremediation efficiency of *B. cereus*, cultivated in 50% POME.

Parameters	**50% POME**		**Removal efficiency*** (%)**
	Before treatment* (mg/L)	**After treatment** (mg/L)**	
Biochemical oxygen demand (BOD)	14,821 ± 984	4,054 ± 368	72.65
Chemical oxygen demand (COD)	23,532 ± 762	4,859 ± 605	79.35
Total organic content (TOC)	3,485 ± 234	861 ± 139	75.29
Total phenolic content (TPC)	1,044 ± 87	320 ± 64	69.34
Ammoniacal nitrogen (AN)	65 ± 9	41 ± 11	36.92
Nitrite nitrogen (NN)	81 ± 12	52 ± 14	35.80
Total nitrogen (TN)	328 ± 18	191 ± 36	41.76
Total dissolved solid (TDS)	19,687 ± 873	6,054 ± 573	69.25
Total solid (TS)	35,085 ± 996	16,223 ± 861	53.76
Total suspended solid (TSS)	14,963 ± 651	5,101 ± 327	65.91
Oil and Grease	3,523 ± 585	910 ± 458	74.17

*The values were obtained after autoclaved of 50% POME; **All analyses were performed after biomass separation from fermented broths; ***The removal efficiency was determined only for average values.

Generally, POME contains huge amounts of organic substances in the form of suspended solids, which are mostly the cellulolytic material originated from cellulose fruit debris (palm mesocarp) (Bala et al., 2015). Therefore, cellulase-producing bacteria

are required for the degradation of cellulose in POME. The presence of organic compounds in POME is responsible for high COD, BOD, and TOC and serves as a suitable substrate for growth of a wide variety of microbes (Lanka & Pydipalli, 2018b). In addition, lipid content (i.e., oil and grease) makes it a suitable medium for several groups of lipase producing microbes as well as hydrocarbon degraders (Ahmad et al., 2018). Some recent reports have shown that *B. cereus* could excrete a cellulase enzyme and thereby facilitate cellulolytic activity in a liquid medium (Bala et al., 2014, 2015). Therefore, the decrease in BOD is attributed to the fact that the *B. cereus* would have reduced the organic load by exploiting cellulolytic activity in POME. Furthermore, *B. cereus* might have degraded the cellulose into reducing sugars by using the cellulases and utilized it as a sole source of carbon during growth. A significant reduction (74.17%) of oil content (oil and grease) in POME was observed which could be attributed to the lipolytic activity of lipase enzyme excreted by *B. cereus* (Singh et al., 2010).

In general, the nitrogen compounds present in organic forms in POME are converted into ammonia with time (Nwuche et al., 2014a). The presence of excessive nitrogen in POME wastewater needs to be reduced since high nitrogen content may lead to eutrophication and toxicity. In the present study, a significant removal of AN, NN, TN was achieved to 36.92%, 35.80%, 41.76%, respectively, as shown in Table 4.1. In the nitrification process, the nitrogenous compound, especially NH_4^+ -N, is usually removed through the heterotrophic nitrification and aerobic denitrification mechanism, while NO_3^- -N is removed by solely aerobic denitrification process. It has been reported that *B. cereus* could facilitate denitrification for removing nitrogenous compounds either in aerobic or anoxic conditions (Rout et al., 2018). In a recent report, Banerjee and Ghoshal (2017) have shown that the strain of *B. cereus* AKG1 reduced 54% NH_4 C-N from petroleum wastewater in a batch mode fermentation process.

4.4 Pollutants removal by *B. cereus*

To observe the bioremediation of POME by *B. cereus*, the removal of the major pollutants (BOD, COD, TOC, TPC) were studied over time (144 h) for 50% POME, as presented in Figure 4.2. Figure 4.2 shows that the pollutants were gradually removed until ~108 h and thereafter reached a plateau. In addition, the remediation of inhibitors was increased as a function of running time, which was positively correlated with the growth

of *B. cereus* in POME (Figure 4.1). The reduction of BOD, COD, TOC, and TPC was mostly achieved within the first ~ 108 h (Figure 4.2), and concomitantly the cell growth reached the maximum at the same time (Figure 4.1). A similar observation was reported by Cheah et al. (2018a), where the higher biomass growth in a medium caused a higher COD reduction. The presence of a lag phase in both Figure 4.1 (first ~ 24 h) and Figure 4.2 indicated a correlation between the biomass growth and pollution removal. This lag phase might be attributed to the adjustment and adaptation period of the bacteria to a new environment, during which time they consumed small amount of nutrients.

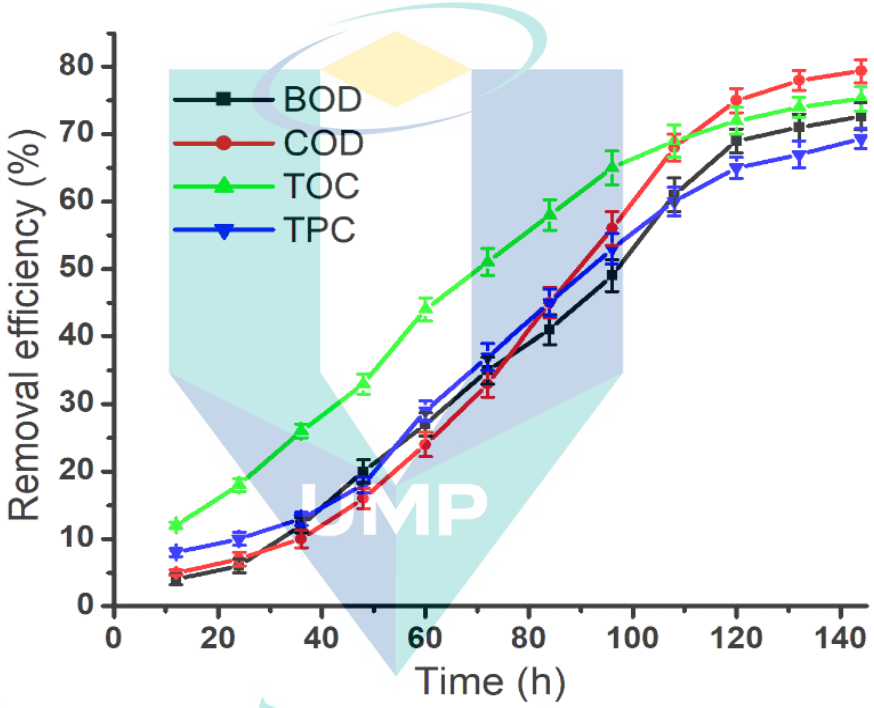

Figure 4.2 The remediation of inhibitors from POME as a function of running time.

The COD and BOD removals were observed to increase gradually (Figure 4.2) during the log phase of bacterial growth (Figure 4.1). A reduction of 79.35% COD and 72.65% BOD could be attributed to the organics degradation because bacteria converted the organics into the simpler form for assimilation (Cheah et al., 2018a). A similar trend was demonstrated in a recent study by Soleimaninanadegani and Manshad (2014), where they found a substantial reduction in COD concentration of POME with an increase in bacterial growth. Nevertheless, the COD and BOD removal efficiency of the present study are in good agreement with those reported by other researchers (Mohammed et al., 2014; Soleimaninanadegani & Manshad, 2014). The TOC removal was found to be 75.29% after 144 h (Figure 4.2) which is significantly higher when compared to some recent reports. The higher TOC removal efficiency could be attributed to the higher

degradation efficiency of hydrocarbons by *B. cereus* as it is capable of excreting hydrocarbon degrading enzymes (Agarry, 2017). Moreover, the degradation of wastewater samples reduced the concentration of phenolics by 69.34%. Banerjee and Ghoshal (2017) observed that *B. cereus* removed 30-83% of TOC and 57% of TPC from petroleum wastewater. It can be observed that the TOC and TPC removal efficiency in the present study is consistent with their study. Apart from the BOD, COD, TOC, and TPC, the wild type *B. cereus* strain (in this study) significantly removed other pollutants, namely, TN, TS, TDS, TSS and oil and grease (Table 4.1). Therefore, the removal efficiency of the present study indicates that *B. cereus* successfully bioremediated POME which could be an effective approach for complex wastewater treatment.

Table 4.2 Comparison of BOD and COD removal efficacy of several *Bacillus* strains from wastewater.

Strains	**Substrates**	**Treatment period (days)**	**BOD removal (%)**	**COD removal (%)**	**References**
Bacillus cereus 103 PB	POME	5	90.98	78.60	(Bala et al., 2015)
Bacillus subtilis 106 PB	POME	5	77.51	64.08	(Bala et al., 2015)
Bacillus cereus	Petroleum refinery effluent	28	-	70.00	(Agarry, 2017)
Bacillus cereus GS-5	Domestic wastewater	13	-	90.60	(Rout et al., 2018)
Bacillus cereus MTCC 25641	Dairy waste effluent	7	44.07	50.68	(Gawai et al., 2017)
Bacillus licheniformis	Municipal wastewater	4	72.08	51.00	(Garode, 2018)
Bacillus cereus KM 15	Orange waste	7	-	62.80	(Qadeer et al., 2018)
Bacillus cereus (MF661883)	POME (50%, v/v)	6	72.65	79.35	Present study

The BOD and COD removal efficiency of this study has been compared with other strains of *Bacillus* and presented in Table 4.2. As displayed in Table 4.1, the efficiency of *B. cereus* (in the present study) is comparable to that of different *Bacillus* spp. reported by some recent studies. The higher COD removal efficiency obtained in the

present study revealed the utilization of a broad range of compounds from the POME wastewater. This could be attributed to the greater tolerance of *B. cereus* to the extremely adverse environmental conditions because of its gram-positive, catalase-positive, and protective endospores forming nature (Yusuf et al., 2013). However, Rout et al. (2018) observed a comparatively higher COD removal efficiency (90.60%), which might be due to a higher retention time (13 days) than that obtained in the present study. Moreover, the bed materials of the packed bed bioreactor, especially dolochar (an activated carbon material), had a high adsorption capacity, which, along with microbial activity, played an important role in removing COD. In another study, Bala et al. (2015) achieved 90.98% of BOD and 78.60% of COD removal efficiency, which could be ascribed to the different substrate concentration and the use of indigenous microbial isolates from POME.

4.5 Survivability of *L. starkeyi* in POME

The survivability and the growth rates of *L. starkeyi* were evaluated using different concentrations of POME (25, 50, 75, and 100%). The growth profiles of *L. starkeyi* are presented in Figure 4.3. Similar growth trends were observed for the broths containing 25, 50, 75, and 100% POME. The highest growth rate was achieved with the broth containing 50% POME, whereas a slightly lower growth was attained with the 25% POME. On the other hand, the growth rate was much lower in the presence of the 100% POME. This effect might be due to the presence of higher phenolic compounds, COD and long-chain fatty acids (Salgado et al., 2016), which may hinder the metabolic power and growth of microorganisms. Additionally, the growth was inhibited at higher substrate concentration (100% POME) might be due to the higher concentration of ammonium as *L. starkeyi* grows in a nitrogen limiting conditions (Yousuf et al., 2010). However, at a higher dilution factor (25% POME), the nutrient concentration was lower and therefore, the microbial growth was reduced. The same phenomenon (reduction of nutrients with dilution) occurred for the 50% POME solution. However, a comparatively higher growth rate was observed in the 50% broth, which may be attributed to the favorable composition (e.g., C/N ratio, lower inhibitors) of the broth (Yousuf et al., 2010).

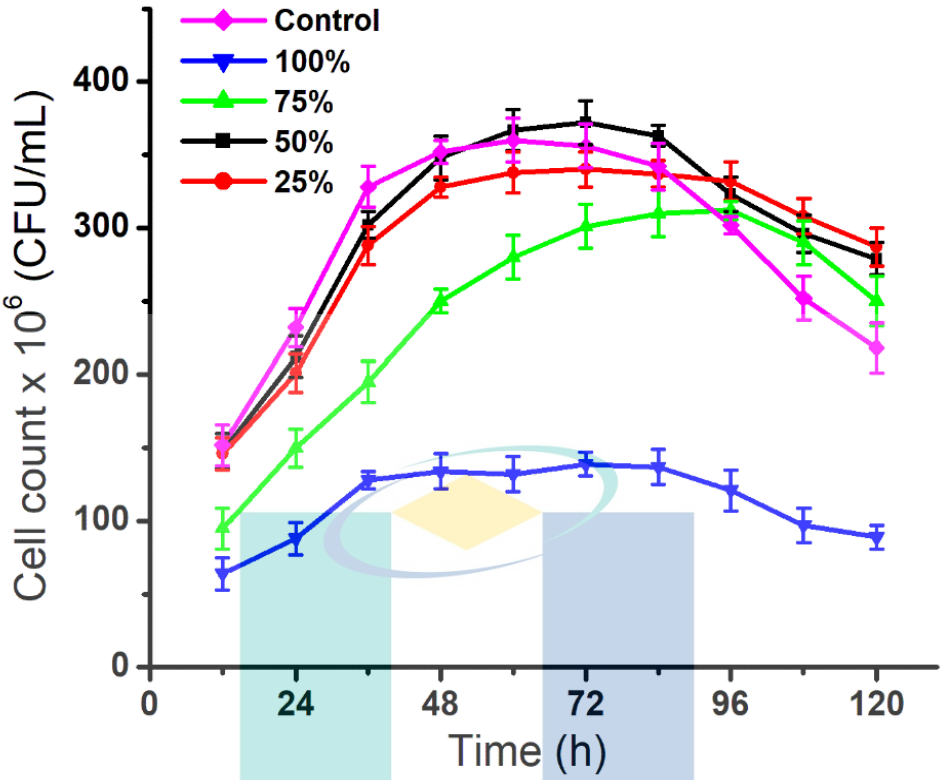

Figure 4.3 Growth profile of *L. starkeyi* in POME.

4.6 POME remediation efficiency of *L. starkeyi*

Raw POME is thick, brownish in color, acidic in nature, with a pH level of ~ 4.5, and consists of 95-96% of water, 4-5% total solids including 2-4% suspended solids and 0.6-0.7% of oil and grease (Ahmad et al., 2016; Chin et al., 2013; Ng et al., 2016). High biological and chemical oxygen demands of POME are the main obstacles to its agricultural uses. On the other hand, POME contains different sugars such as arabinose, xylose, glucose, galactose and mannose, which can serve as potential carbon sources to the microbial community. Apart from that, it is high in nutrients and essential minerals such as K, N, Mg, Ca, P, Fe, B, Zn, Mn and Cu, which support plant growth (Ng et al., 2016) as well as microbial growth (Yousuf et al., 2010). However, the presence of inhibitors such as phenolic compounds, tannic acid (Nur, 2012), acetic and formic acids (Nwoko et al., 2010) make it phytotoxic.

Table 4.3 Wastewater analysis of 50% POME (the removal efficiency was calculated only for average values).

Parameters	50% POME		Removal, %
	Before treatment, mg/L	After treatment, mg/L	
Chemical oxygen demand (COD)	26,176 ± 838	6,541 ± 456	75.01
Total organic content (TOC)	4,230 ± 528	1,423 ± 214	66.35
Biochemical oxygen demand (BOD)	11,025 ± 722	5,243 ± 510	52.44
Ammoniacal nitrogen (AN)	31 ± 2	8 ± 1	74.19
Total phenolic content (TPC)	2,744 ± 118	1,033 ± 94	62.36
Nitrite nitrogen (NN)	86 ± 5	41 ± 9	52.32
Total nitrogen (TN)	305 ± 16	141 ± 17	53.77
Total dissolved solid (TDS)	13,160 ± 526	5,669 ± 413	56.92
Total solid (TS)	26,814 ± 722	16,184 ± 211	39.64
Total suspended solid (TSS)	11,970 ± 512	5,784 ± 115	51.68
Oil and Grease	3,310 ± 423	1,674 ± 720	49.43

The bioremediation of POME by oleaginous yeast was evaluated and nitrogen, COD, TOC, BOD, AN, TPC, NN, TDS, TS and TSS were analyzed. The variation of these parameters in untreated and treated POME is presented in Table 4.3. As shown in Table 4.3, there was a remarkable reduction in COD (75.01%) after 120 h of retention time, indicating that *L. starkeyi* utilized and hydrolyzed most of the organic compounds. A significant number of phenolic compounds (62.36%) was removed by this treatment attributed to the fact that the organic compounds composed of phenolic, acidic and heterocyclic compounds in the POME, were degraded by *L. starkeyi* through aerobic digestion. The decrease in BOD by 52.44% suggests that *L. starkeyi* can reduce the organic load in POME. The changes in ammonia content depend on the transformation of organic nitrogen into inorganic nitrogen. In this aerobic digestion, macromolecular organic matter was significantly degraded to small-molecule organic matter, and therefore, AN and NN reduced by 74.19% and 52.32%, respectively.

4.7 Removal of inhibitors by *L. starkeyi*

To study the bioremediation of POME by oleaginous yeast, three major components COD, TOC and TPC were considered. Generally, POME contains higher levels of COD that is predominantly related to the organic load in the form of TSS, VSS,

TS, oil and grease (Bala et al., 2015). The results presented in Figure 4.4 are recorded from the aerobic digestion of the 50% POME because the microbial growth was at its maximum at this concentration. As shown in Figure 4.4, treatment of *L. starkeyi* led to a ~75% reduction of COD. The COD removal efficiency by *L. starkeyi* has been compared (Table 4.4) to those of other species (bacteria, fungi), and the findings showed similar reduction efficiency. Thus, the considerable COD reduction suggests that the treatment protocol can be practically applicable for the bioremediation of POME.

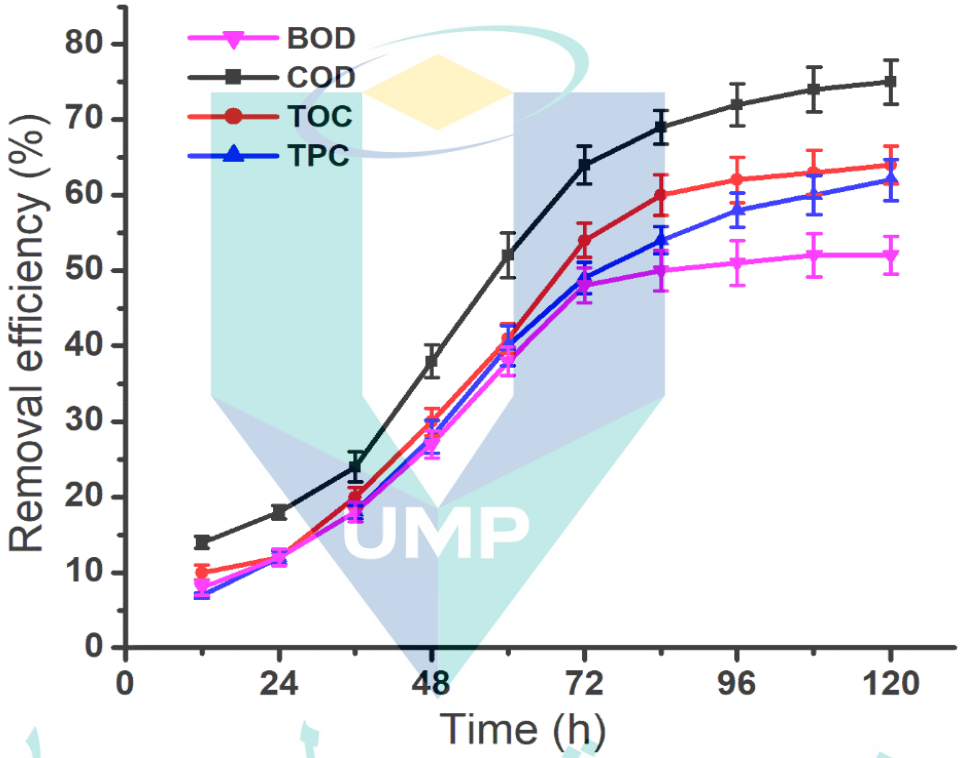

Figure 4.4 Removal efficiency of inhibitors presence in POME.

The carbon content of wastewater is composed of a variety of organic compounds in various oxidation states (Pirozzi et al., 2013). For further use of any industrial wastewater, the TOC must be reduced. In this study, TOC removal of up to ~ 66% was achieved (Figure 4.4). Microbial biomass growth is directly related to TOC reduction (Yousuf et al., 2010) since yeasts consume sugars and other carbon compounds (proteins and phenols) as nutrients. The reduction of TOC was mostly achieved in the first 90 h, i.e., organic carbon was consumed as the primary carbon nutrient until the biomass reached the stationary phase (Figure 4.3). At the same time, the trend in the reduction of TPC was similar to that of TOC, which indicated that *L. starkeyi* is a suitable species to consume or reduce the phytotoxic compounds (mainly phenols). The removal of phenolic

compounds was further confirmed by the increase in pH over time (Figure A1). Generally, POME contains phenolic compounds such as caffeic acid, ferulic acid, 4-hydroxybenzoic acid, catechol, and 3-methylcatechol, which are mainly responsible for the lower pH of POME (Khongkhaem et al., 2016). The increase in pH (Figure A1, in appendix) might be due to the consumption or breakdown of phenolic compounds of POME by *L. starkeyi*, which leads to the formation of hydroxide ions (Louhasakul et al., 2016). Similar results have been reported in a study where they observed an increase in pH from 3.98 to 7.79 when POME was treated with a mixed bacterial culture. The success of such treatments depends on several factors, such as the type of micro-organisms, composition of effluents, and the concentration of TPC for the adaptation of selective consortia Therefore, this study provides an alternative way to valorize the POME as a growth media for the production of microbial lipids suitable for biodiesel production and simultaneously provides a strategy to reduce wastewater pollution loads.

Table 4.4 Comparison of COD removal efficiencies of microorganisms from POME.

Group	Species	Initial COD (mg/L)	Treatment period (days)	COD removal (%)	References
Bacteria	*Micrococcus luteus*	75,900	5	67.19	(Bala et al., 2015)
	Stenotrophomonas maltophilia			61.92	(Bala et al., 2015)
	Bacillus cereus			78.60	(Bala et al., 2015)
	Bacillus subtilis			64.08	(Bala et al., 2015)
	Anaerobic sludge (Mixed culture)	75,000	7	45.00	(Tabassum et al., 2015)
Yeast	*Yarrowia lipolytica*	11,000	2	95.00	(Oswal et al., 2002b)
	Yarrowia lipolytica	37,000	3	72.90	(Louhasakul et al., 2016)
	Saccharomyces sp. L3[1]	114,800	4	83.00	(Iwuagwu & Ugwuanyi, 2014)
	Candida rugosa CU1	-	6	54.70	(Theerachat et al., 2017)
	Lipomyces starkeyi	26,176	6	75.01	Present study

4.8 Biomass growth profile for co-culture of *B. cereus* and *L. starkeyi*

The 50% POME (the optimal concentration for *B. cereus* and *L. starkeyi*, as shown in the 4.2 and 4.5 sections) sample was used as growth medium for the co-culture of *B. cereus* and *L. starkeyi*. The growth kinetics of *B. cereus*, *L. starkeyi* and co-culture were studied using 50% POME as presented in Figure 4.5 and microbial cells were visualized in Figure 4.6. As can be seen from Figure 4.5 that the cell growth kinetics followed similar trend for all inoculums where they reached a plateau after 72 h and started decaying after 100 h. The maximum growth was achieved for co-culture inoculum than the mon-culture of *B. cereus* and *L. starkeyi*.

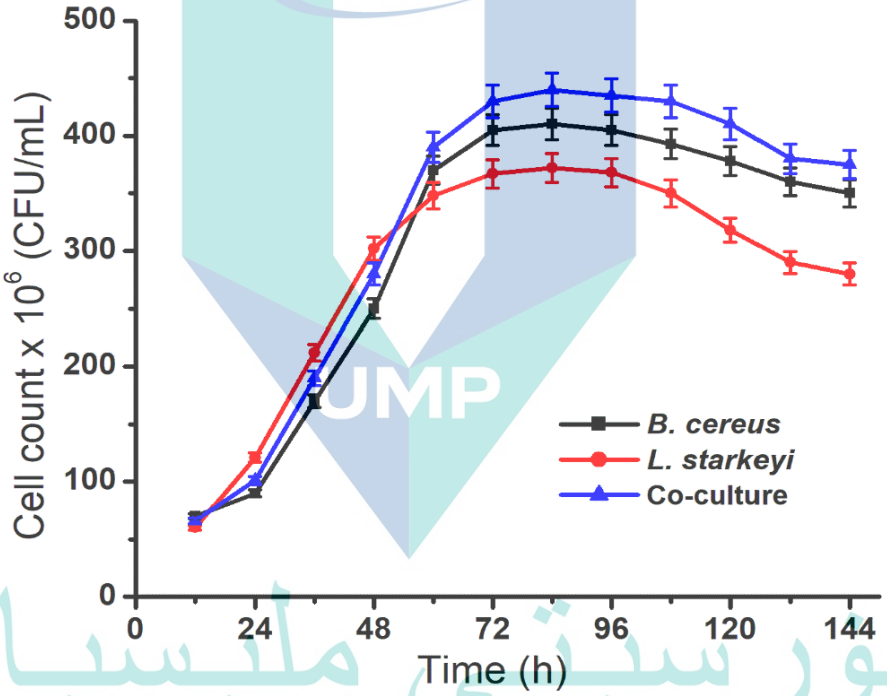

Figure 4.5 The growth profile of *B. cereus*, *L. starkeyi*, their co-culture inoculum in the 50% POME, at a temperature of 30 ºC.

The maximum growth was achieved for co-culture inoculum, although the initial growth rate for *L. starkeyi* was slightly higher than the co-culture and *B. cereus* monoculture inoculum. The highest growth observed in the co-culture inoculum might be accredited to the combined synergistic effect of both bacteria and yeast combinations during the treatment process. The synergistic interactions between these two microbes enabled the microorganisms to maximize their metabolic abilities and to maintain a community integrity and stability (El-Masry et al., 2004). Thus, a higher biomass growth was achieved for co-culture than the monocultures. Nevertheless, *B. cereus* has been

shown to achieve higher biomass growth than *L. starkeyi*. This may be explained by greater tolerance of *B. cereus* to high pollutant concentrations due to their wider enzymatic potentials and spore forming ability (Asadi et al., 2020). On the other hand, *L. starkeyi* were reported to have ability to assimilate oil and grease present in POME, making POME more favourable for bacteria cultivation (Ahmad et al., 2019). The synergistic effect of mixed microbial consortium of organisms has also been reported elsewhere in the literatures (Benka-Coker & Ekundayo, 1997; Chigusa et al., 1996; Zhang et al., 2014a).

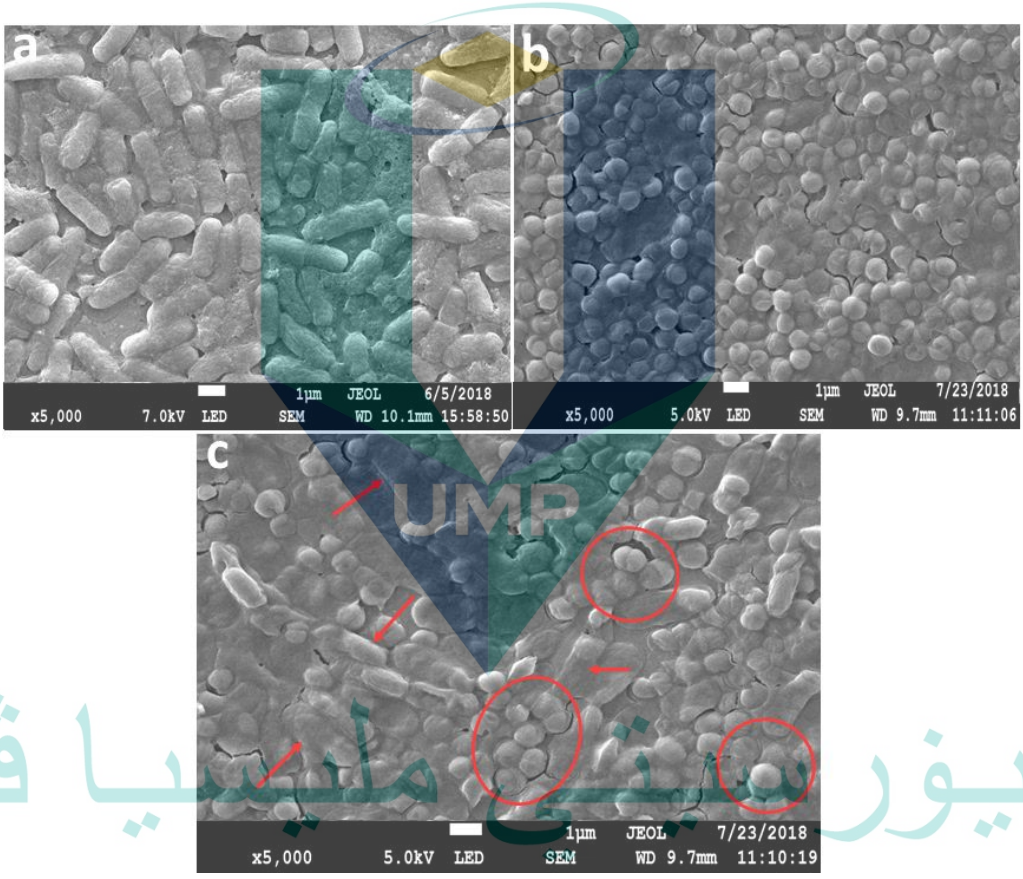

Figure 4.6 Visualization of different inoculums by FESEM image in the POME. (a) *B. cereus*, (b) *L. starkeyi*, (c) *B. cereus* and *L. starkeyi* co-culture (red circles show yeast cells and red arrows indicate bacteria cells).

4.9 Remediation of inhibitors by co-culture

The 50% POME sample was characterized by analyzing BOD, COD, TOC, TPC, AN, NN, TN, TSS, TS, and TDS before and after treatment by the co-culture inoculm. The variation of these parameters in untreated and treated POME is presented in Table 4.5.

Table 4.5 Bioremediation efficiency of co-culture consortium cultivated in the 50% POME.

Parameters	50% POME		
	Before treatment (mg/L)	**After treatment (mg/L)**	**Removal efficiency* (%)**
Chemical oxygen demand (COD)	25,825 ± 1,135	4,219 ± 456	83.66
Biochemical oxygen demand (BOD)	17,390 ± 965	3,940 ± 214	77.34
Total phenolic content (TPC)	1,380 ± 114	374 ± 84	72.89
Total organic content (TOC)	4,132 ± 510	771 ± 117	81.34
Total nitrogen (TN)	464 ± 64	161 ± 94	65.30
Ammoniacal nitrogen (AN)	94 ± 18	22 ± 9	76.59
Nitrite nitrogen (NN)	128 ± 43	40 ± 17	68.75
Total solids (TS)	16,022 ± 789	5,500 ± 413	65.67
Total suspended solids (TSS)	7,124 ± 656	2,035 ± 211	71.43
Total dissolved solids (TDS)	11,634 ± 804	2,832 ± 115	75.66
Oil and grease	4,031 ± 529	837 ± 320	79.23

*The removal efficiency was calculated only for average values.

The COD removal efficiency is presented in Figure 4.7. As can be seen in Figure 4.7, the removal of COD was increased as a function of running time, which was positively correlated with the growth of microbial cells in POME (Figure 4.5). The COD reduction of 79.35 ± 1.7%, 75.01 ± 2%, and 83.66 ± 3% of COD was achieved for 144 h treatment of POME by *B. cereus*, *L. starkeyi*, and co-culture inoculum, respectively. This was due to the degradation of organics in the POME by microbial assimilation because the organics converted into the simpler form for microbial assimilation (Cheah et al., 2018a).

The COD removals were observed to increase gradually during the log phase (~ 72 h) of microbial growth (Figure 4.5). The reduction of COD was mostly achieved within the first ~ 96 h (Figure 4.7), and concomitantly the cell growth reached the

maximum at the same time (Figure 4.5). The COD removal was higher for *B. cereus* than *L. starkeyi*, might be due to the greater tolerance of *B. cereus* to the extremely adverse environmental conditions because of its gram-positive, catalase-positive, and protective endospores forming nature (Yusuf et al., 2013). The maximum COD removal efficiency obtained by co-culture could be attributed to the utilization of a broad range of compounds from the POME wastewater because it has been reported that yeast can degrade certain type of substrates while bacteria can assimilate other type of substrates and therefore combination of them accelerated the degradation efficiency of POME which is composed of several simple and complex substrates.

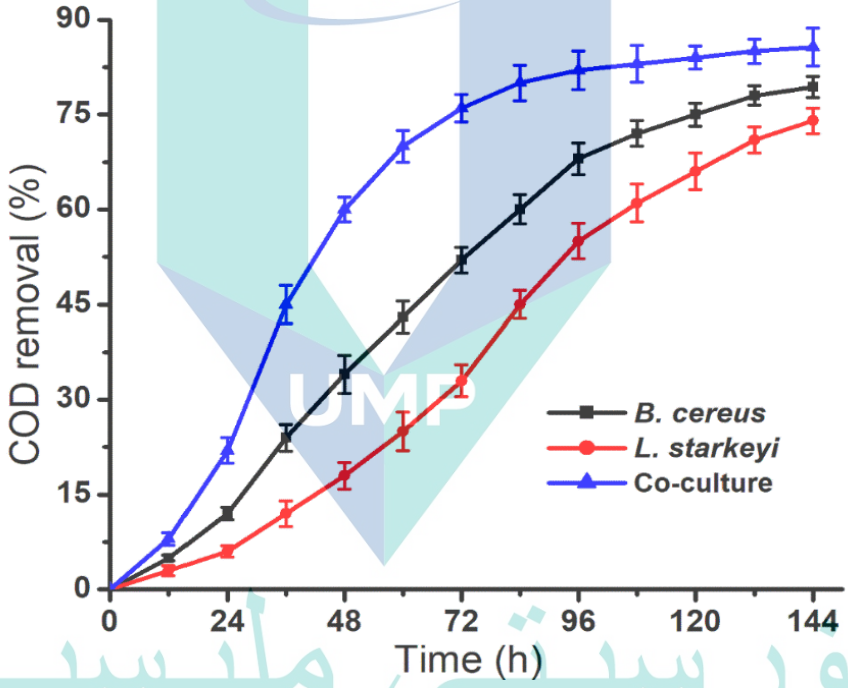

Figure 4.7 Removal of COD from POME by *B. cereus*, *L. starkeyi*, and their co-culture from POME as a function of running time.

Consequently, a comparatively higher biomass growth was achieved for co-culture than monocultures (Figure 4.5), which also led to a higher COD removal (Cheah et al., 2018a). This observation is corroborated by a recent study of Cheah et al. (2018a), where the higher biomass growth in a fermentation medium caused a higher COD reduction in POME. Cheirsilp et al. (2011) reported that the biomass concentration in the mixed culture of yeast *R. glutinis* and microalgae *C. vulgaris* increased faster and was higher compared to that in the pure cultures. After day 4, the cell growth was observed to decrease and there was no significant enhancement in biomass production. This might

be resulted from the depletion of the nutrients in the culture medium. The COD removal remained almost unchanged after four days in the co-culture, attributed to the fact that the consumable carbon source present in the effluent was limited, namely not all of COD could be consumed by the yeast and bacteria. Van Hamme et al. (2000) demonstrated that such mixed culture combination displays metabolic versatility and superiority to pure cultures. Consequently, a microbial consortium containing a number of microorganisms could be considered to be well suited for the degradation of industrial wastewaters (Sathishkumar et al., 2008). Similarly, Sugiura et al. (1996) and Sathishkumar et al. (2008) reported that the mixed microbial combination showed the maximum reduction of organic load from wastewater.

Table 4.6 Performance of different co-culture for COD removal from wastewater.

Strains	**Co-culture consortia**	**Substrates**	**Time (days)**	**COD removal (%)**	**References**
Pseudomonas sp. on *C. sorokiniana* CY-1	Microalgae-bacteria, 1:1	30% (v/v) POME	5	53.70	(Cheah et al., 2018c)
K. variicola and *P. aeruginosa*	Bacteria-bacteria, 1:1	50% (v/v) POME	11	69.28	(Islam et al., 2018b)
B. cereus 103 PB and *B. subtilis* 106 PB	Bacteria-bacteria, 1:1	POME	5	90.64	(Bala et al., 2015)
R. gultinis and *C. vulgaris*	Yeast-microalgae, 1:1	Sugar cane plant wastewater (molasses)	7	79.00	(Cheirsilp et al., 2011)
B. cereus and *L. starkeyi*	Bacteria-yeast, 1:1	50% (v/v) POME	6	83.66	Present study

The COD removal efficiency of this study has been compared with other co-culture inoculums and is presented in Table 4.6. As displayed in Table 4.6, the efficiency of *B. cereus* and *L. starkeyi* (in the present study) is comparable to that of different co-culture consortiums reported by some recent studies. The higher COD removal efficiency obtained in the present study revealed the utilization of a broad range of compounds from the POME wastewater (Figure 4.8). However, Bala et al. (2015) obtained a relatively higher COD removal (90.64%) using a co-culture of *B. cereus* 103 PB and *B. subtilis* 106 PB, which could be ascribed to the higher inoculum concentration. Another reason could

be the use of the indigenous microbial isolates from POME i. e., the bacterial strains used in their study were isolated from POME. In another study, De Felice et al. (1997) used a combination of bacteria and yeast to degrade olive oil mill wastewater. The microbial combination reduced the COD of olive oil mill wastewater by 80% in their study.

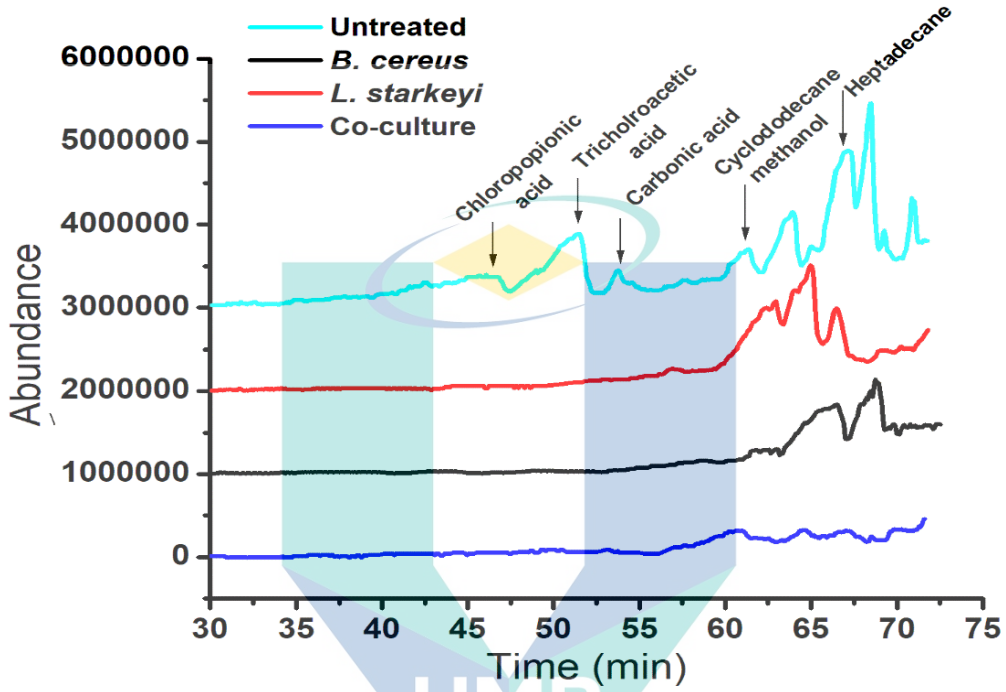

Figure 4.8 GC-MS peak for POME after 6 days of treatment by *B. cereus*, *L. starkeyi*, and their co-culture.

The GC-MS data (Figure 4.8) indicate that organic compounds in the effluent were mainly composed of phenolic (cyclododecanemethanol), acidic (trichloroacetic acid, carbonic acid, 2-chloropropionic acid) and heterocyclic compounds (1-cyclopentyl-4-1-methylethylcyclohexane, cyclohexane 3-3-dimethyl-5-oxo), which were completely degraded by the co-culture through aerobic digestion. Thus, a significant number of phenolic compounds (72.89%) was removed by this treatment. The decrease in BOD by 77.34% suggests that the co-culture inoculum was able to reduce the organic load in POME. The changes in ammonia content depend on the transformation of organic nitrogen into inorganic nitrogen. In this aerobic digestion, macromolecular organic matter was significantly degraded to small-molecule organic matter, and therefore, AN and NN reduced by 76.59% and 68.75%, respectively. It can be concluded that the synergistic effect of mixed microbial consortium of microorganisms for POME treatment brings enhanced performance for effective biodegradation.

4.10 The degree of bioremediation

The degree of bioremediation (after 6 days) was studied by determining seed germination profiles of Mung beans (*V. radiata*) (Kumar & Singhal, 2009), as presented in Figure 4.9a and Figure A2 (in appendix). A significant difference in germination was observed (Figure 4.9a) when the seeds were grown in different medium of POME. The number of germinated seeds increased gradually with time until 60 h for all samples, and then it reached a plateau. The maximum seed germination occurred in co-culture treated POME after 3 days of observation, whereas the minimum germination was observed in raw POME. Consequently, the highest GI was obtained for co-culture treated sample followed by *B. cereus* and *L. starkeyi* treated samples (Figure 4.9b).

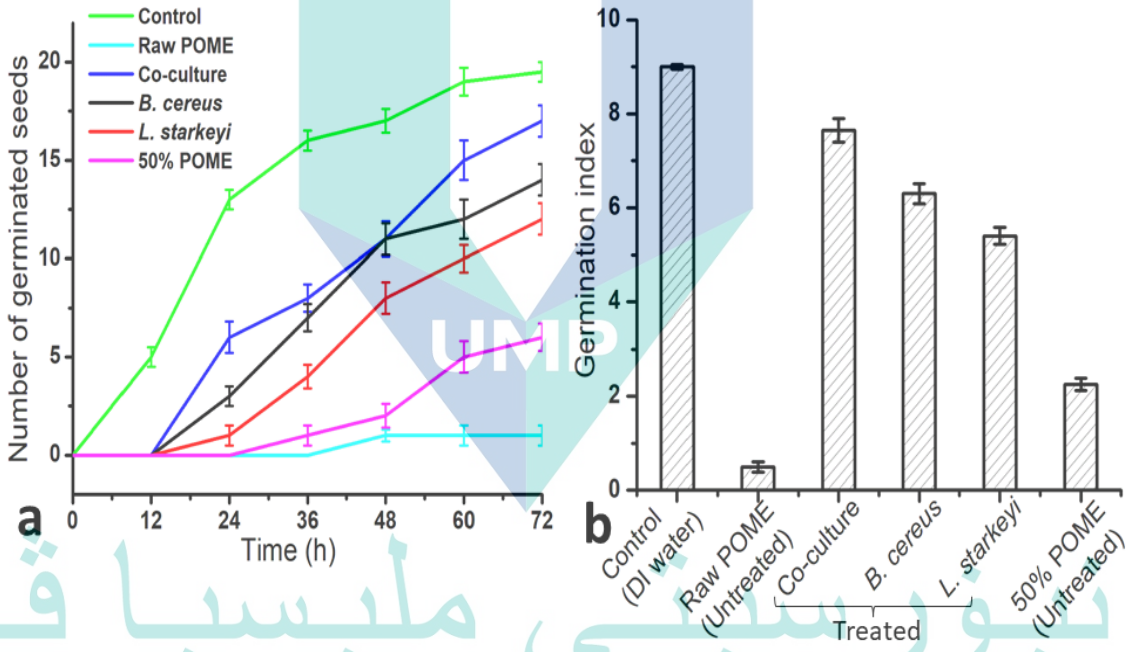

Figure 4.9 a) The profiles of seed germination in untreated (raw POME and 50% POME) and treated POME medium (by *B. cereus*, *L. starkeyi*, and their co-culture); b) Germination index.

The reduced germination in untreated POME samples (raw POME and 50% POME) was possibly due to an imbalance between toxic compounds and nutrients. This lower germination was found in POME samples, possibly due to the presence of higher organic and toxic compounds. However, in lower POME concentrations i.e., in 50% POME, the higher GI (Figure 4.6b) and germination visibility values (Figure A2, in appendix) indicating the toxicity reduced mainly due to dilution. The higher organic load, low pH, high nitrogen content, and contaminants negatively influenced the plants' growth

(Li et al., 2017). In addition, concentrated POME is not favorable for the growth of plants due to the presence of more phenolic compounds (Table A1, in appendix), which include various acids (i.e., caffeic acid, ferulic acid, 4-hydroxybenzoic acid) (Liu et al., 2016), and therefore, a lower germination rate was observed in untreated POME. The higher GI in treated POME confirmed that the inhibiting agents were successfully degraded by *B. cereus*, *L. starkeyi*, and their co-culture. Higher GI, indicating bioremediation of phytotoxic compounds by microbial assimilation. The highest GI was achieved for the sample treated with co-culture attributed to the better removal of phytotoxic compounds (Figure 4.9). Similar observations to this finding were reported by Pandey et al. (2008), and they found that the high concentration of brewery and distillery effluent had an inhibitory effect on the seed germination and growth of maize and rice plants at the early stage of plantation. Likewise, to this study, Ogunwenmo et al. (2010) reported that the seed germination of *Amaranthus hybridus* was enhanced in treated brewery effluent. Therefore, it can be concluded that the diluted POME treated with *B. cereus*, *L. starkeyi*, and their co-culture could be deployed into the arable land since it would not be detrimental for plant growth. This treated effluent could be used both for invigorating the seeds and for further irrigating crops or nurseries.

The overall findings of this research work suggest that a co-culture of *B. cereus* and *L. starkeyi* could be a potential inoculum for bioremediation of POME. It was observed that among the different dilutions, the moderately dilute solution of POME (50%) showed higher microbial growth and offered a significantly higher degree of bioremediation. Furthermore, higher remediation was achieved using the co-culture inoculum in the 50% POME. The COD reduction of 79.35 ± 1.7%, 75.01 ± 2%, and 83.66 ± 3% of COD was achieved for 144h treatment of POME by *B. cereus*, *L. starkeyi* and co-culture inoculum, respectively. Because, a higher degradation of pollutants and toxic components of POME could be achieved by co-culture inoculum. This finding was further confirmed by determining the seed GI of *V. radiata*. POME treated with co-culture displayed higher GI values than the untreated samples due to the significant remediation of detrimental organics present in the POME. This approach of wastewater treatment by microbes could be beneficial due to the easier cultivation; diverse inexpensive carbon sources and carbon utilization pathways; rapid growth rate; higher biomass production capacity. Therefore, treatment of POME by microorganisms would be an attractive option for wastewater treatment and ultimately, environmental resilience.

CHAPTER 5

MICROBIAL LIPID EXTRACTION BY USING ELECTROPORATION AND COMPARISON WITH DIFFERENT CONVENTIONAL METHODS

5.1 Introduction

In this chapter, a lab scale electroporation device was used to evaluate the potentiality of EP technique on yeast cell (*L. starkeyi*) wall disruption and lipid extraction. The cell wall disruption was confirmed by visualizing in FESEM image. The efficiency of EP was evaluated by calculating cell inactivation rates and measuring extracted lipid from wet biomass of *L. starkeyi*. Furthermore, the performance of lipid extraction by EP technique was compared with other methods to validate its applicability.

5.2 Cell inactivation of *L. starkeyi* cell by electroporation

Recently, EP was proposed as a new cell disruption approach to extract lipid from microbial biomass for the direct transesterification process to produce biodiesel (Yousuf et al., 2017b). In this technique, the high electric pulses of DC are applied to living cells and tissues for a short duration of time to permeabilize the cell membrane for transfection or transformation (Faridnia et al., 2015; Luengo et al., 2015). These pulses are delivered to a pair of electrodes by a pulse generator (Garoma & Shackelford, 2014). Basically, a membrane potential is induced by an externally applied electric field (Kotnik et al., 2015). However, irreversible EP can damage the cell wall thus permeabilizing the cells after being subjected to high voltage pulses with sufficient strength and time of treatment (Yano et al., 2017).

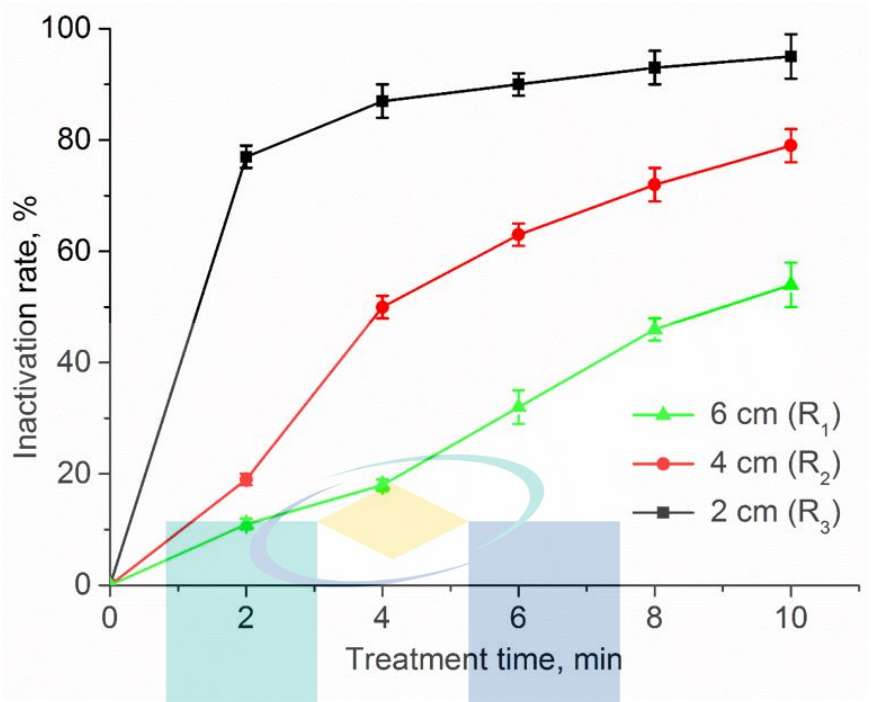

Figure 5.1 Effect of EP on cell inactivation using different electrode distances.

The effect of EP on cell inactivation is presented in Figure 5.1 which illustrates that the cell inactivation was gradually increased until 8 min and thereafter no significant changes were observed for all reactors. Initial inactivation rate was very fast for the R_3, where it achieved 77% inactivation within 2 min (Figure A3, in appendix). At the same time, R_1 and R_2 inactivated only 10% and 18% cells, respectively. Although, R_1 and R_2 showed almost a constant increasing rate of inactivation, after 10 min they achieved up to 54% and 79% inactivation correspondingly. Whereas, more than 95% cells were inactivated in R_3 within 10 min. The higher cell inactivation in R_3 demonstrates that the high pulse electric field on the cell would have damaged the cell wall of yeast thus enhanced more dead cells in the reactor. Since, the high-voltage electrical pulses of short duration (0.01 s) were applied to induce irreversible permeabilization of the cell wall, probably through nanoscale defects in the outer layer, leading to cell wall disruption (Pillet et al., 2016). Related study also stated that the EP treatment has strong effect on cell viability and changes in cell wall structure, leading to increased wall porosity (Ganeva et al., 2014). Therefore, the higher electric pulses could directly attack the basic building blocks of yeast cell wall (β(1→3)-glucan, β(1→6)-glucan, chitin, and mannoproteins) thus inactivate the yeast cells and enhances the release of intracellular biomolecules (Flisar et al., 2014; Kollár et al., 1997).

5.3 Cell disruption visualization

To observe cell wall break- up, yeast cells were visualized by FESEM as presented in Figure 5.2. The undisrupted cell in absence of EP treatment is displayed in Figure 5.2a, while, ruptured cells (red circles) are clearly observed after 10 min of EP treatment as shown in the Figure 5.2b. The cell wall break-up that was achieved might be due to the higher TI (36.7 kWh/m^3) of EP. The mechanism of cell disruption during EP treatment was described by Sheng et al. (2011), where electroporation created electrical charge on the dielectric cell wall thus caused irreversible breakdown of the microbial cell.

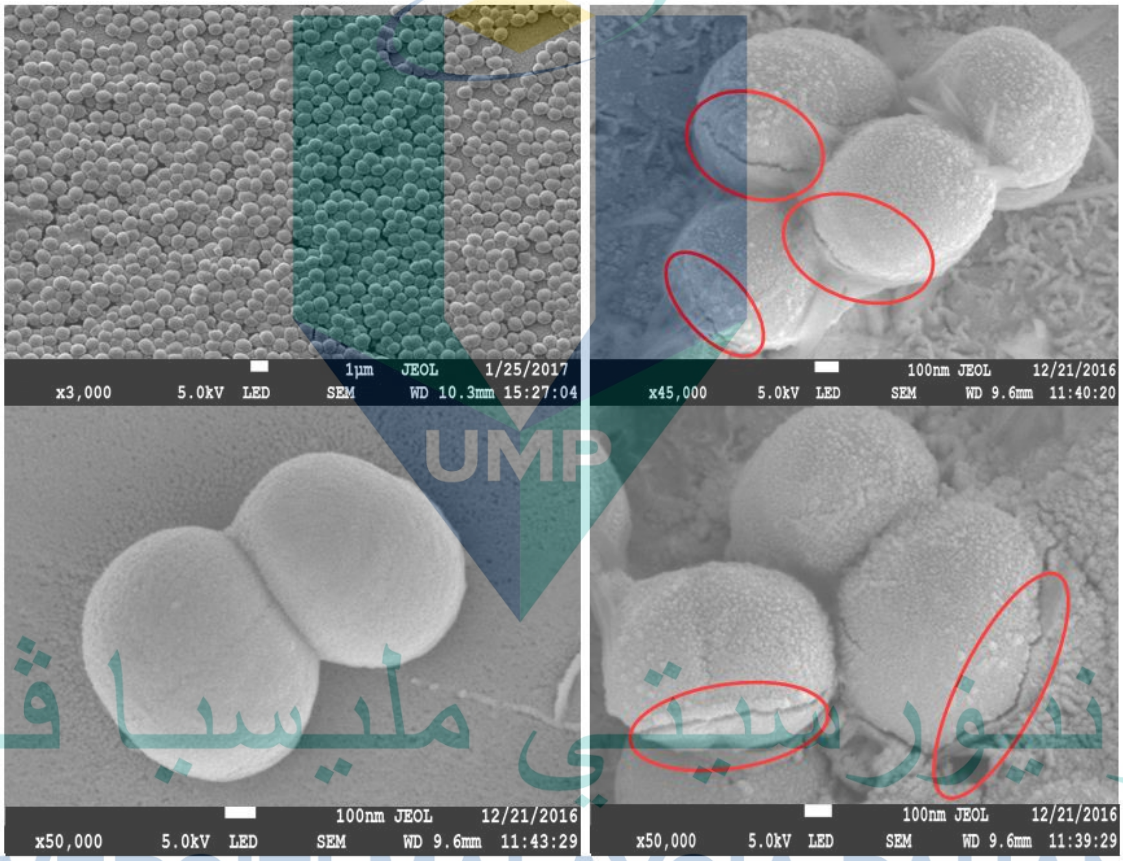

Figure 5.2 FESEM image of *L. Starkeyi* (a) before and (b) after EP treatment, the disrupted cells are indicated by red circles.

5.4 Lipid extraction and quantification

The performance of EP to extract lipid from the yeast cells is presented in Figure 5.3 and Figure A4 (in appendix). Three different distances (2, 4 and 6 cm) between two electrodes were considered with different treatment time (2, 4, 6, 8 and 10 min). The lipid

extraction was increased sharply up to 6 min for all electrode distances; however, further increment of time did not show any significant enhancement. The R_1 achieved maximum lipid of 13 mg/g after 10 min EP treatment, whereas R_2 obtained 41 mg/g, which is around three times higher than R_1. On the other hand, maximum performance (63 mg/g) was observed in R_3 compared to others (R_1 and R_2).

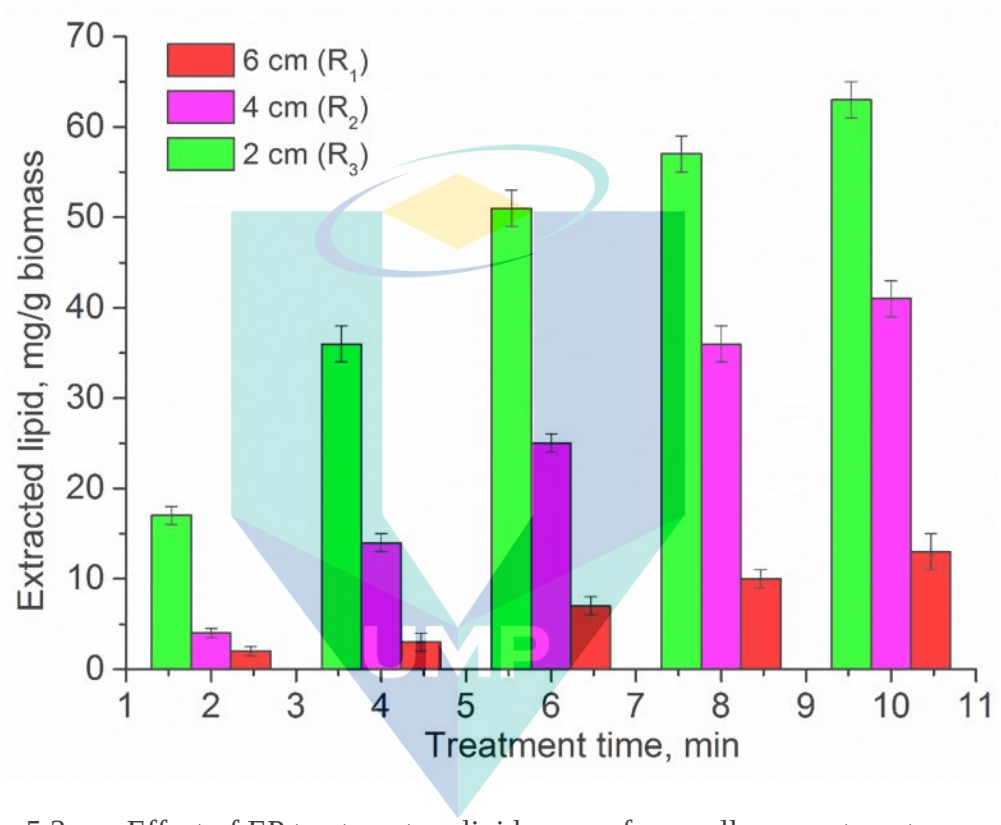

Figure 5.3 Effect of EP treatment on lipid escape from cell compartment.

A significant amount of lipids are trapped in the cytoplasm by intact cell walls; hence, the lipid extraction efficiency greatly depends on the extent of cell disruption (Ren et al., 2017). As a result, at 2 cm distance the cell disruption was higher and led to a higher amount of lipid extraction. The less distance exhibited more cell disruption as well as lipid extraction because of higher intensity of the electric field (Table. 5.1). Sheng et al. (2011) reported similar observation in their study, where EP treatment intensity was directly correlated with microbial cell wall disruption as well as lipid extraction. They also observed that the severity of damage of cell wall grew by increasing TI and, in turn, lipid recovery was enhanced. In their study, the maximum number of cells were disrupted with a TI value of 35.8 kWh/m^3, which is almost equal to the value used in this study (36.7 kWh/m^3). Cell disruption by EP treatment at this TI value may produce a less resistant barrier towards the intrusion of organic solvent. As a result, the biomass pellet

would be more homogeneously contacted with organic solvent after centrifugation, which could accelerate the rate of lipid extraction. In addition, the rate of extracted lipid was also increased (Figure 5.3) because of the increment of TI value (Table 5.1) with treatment time for all electrode distances.

Table 5.1 Variation of TI with respect to time and distance between electrodes.

Time, min	Treatment Intensity, kWh/m^3 at different distances between electrodes		
	2 cm	4 cm	6 cm
0	0	0	0
2	7.3	1.8	0.8
4	14.7	3.7	1.6
6	22.0	5.5	2.4
8	29.3	7.3	3.3
10	36.7	9.2	4.1

To break up the cell wall, generally several physical methods such as thermolysis, osmotic shocks, laser treatments and ultrasound have been considered as viable physical methods with the aim of lipid extraction; however, these methods are associated with high-energy consumption (Steriti et al., 2014). Recently, several chemical methods (i.e., Fenton's, chloroform: methanol= 2:1, Bligh and Drier) have gained attention for lipid extraction due to their lessened energy and time consumption. However, these methods still have some significant limitations. In particular, chemicals must be continuously supplied, and this aspect might greatly affect the economic sustainability of the technology when large scale production systems are considered. Moreover, exhaust disrupting solution should be treated before being disposed as liquid waste. Furthermore, acids and alkalis might corrode the equipment surface and attack the valuable products (i.e., lipids) of the microbial cell, thereby detracting from the effectiveness of the entire process. Several cell wall disruption techniques such as solvent (methanol: chloroform; 2:1), Fenton's method, ultrasound for lipid extraction were studied to compare the efficiency of EP over those methods.

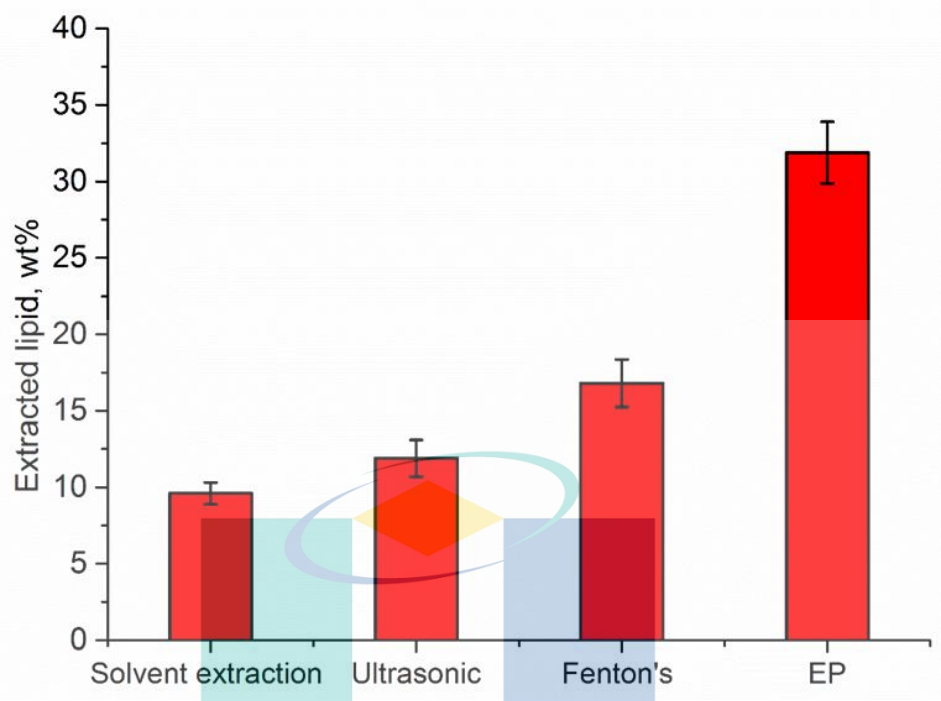

Figure 5.4 Lipid extraction (wt%, dry biomass) from different type of cell disruption methods.

The amount of extracted lipid that was obtained by using different cell wall disruption techniques is shown in Figure 5.4. It clearly shows that the maximum lipid (31.88%, wt.%) was extracted for EP treatment whereas minimum amount (9.6%) was obtained for solvent extraction. The lower lipid extraction was achieved might be due to less cell disruption efficiency by solvent extraction technique. Moreover, the lipid content might be reduced in most of the chemical processes because of lipid degradation by further chemical reaction. The ultrasonic and Fenton's techniques achieved 11.89% and 16.8% lipid recovery, which were about 2 and 2.5 times lower than EP.

The efficiency of ultrasound for lipid extraction was analyzed and maximum of 11.89% lipid recovery was achieved using this technique. Ultrasound (35 kHz) has been applied to yeast to disrupt the cell wall and extract lipid from the cell. The application of ultrasound to yeast in water, also known as sonication, utilizes the process of cavitation to disrupt the cell wall (de San & Parres, 2014). The low frequency ultrasound exposes the cell wall of yeast to damaging shear forces that promote the release of intracellular compounds. Generally, high pressure (300 MPa at 25 °C for 10 min) and temperature (50 °C for 60 min) are required to destroy the cell wall of microorganisms and liberate lipids to the extraction medium (Gonzalez & Barrett, 2010). Therefore, in the present study, the

lower lipid extraction that was achieved compared to Fenton's and EP techniques might be due to using low temperature (27 ± 2 °C) and atmospheric pressure (1 atm) (Feng et al., 2008). The study of lipid extraction from *Yarrowia lipolytica* yeast by Meullemiestre et al. (2016) also supports this statement. In solvent extraction, chloroform and methanol (2:1), commonly known as Folch method (Folch et al., 1957), were employed for cell disruption because a combination of polar and non-polar solvents could extract more lipids than when individual solvents are used (Ryckebosch et al., 2012). In this co-solvent system, chloroform and methanol form a co-mixture solvent that dissolves the lipids. The chloroform dissolves the neutral lipids (triglycerides) while the methanol dissolves the polar membrane lipids (Cooney et al., 2009). However, only 9.6% lipid could be obtained for solvent extraction in this study. The solvent extraction alone was less efficient, and as a result, less lipid extraction was achieved compared to all other methods. In addition to other challenges involved in the recovery and conversion of microbial lipids in this process, the high water content of the biomass can lower both the esterification reaction rate and the lipid transfer from the microbial cell to the reaction medium (Yousuf et al., 2016).

Generally, the recovery of lipid from the microbial biomass is challenging especially when wet biomass is used for lipid extraction because the high-water content of the biomass can lower the lipid transfer from the microbial cell to the reaction medium (Yousuf et al., 2017b). Usually, more solvent is used to obtain higher lipid recovery and higher conversion of lipids to FAME (Sheng et al., 2011). Cheirsilp and Louhasakul (2013) conducted direct transesterification without adding a nonpolar solvent and the reaction time was longer (6 h) with a methanol/biomass ratio of 125:1. However, with the increase of methanol/biomass ratio to 209:1, the reaction time was reduced to 1 h. In another study, Wahlen et al. (2011) directly transesterified wet microalgae cells and found that the wet cells with a water content of more than 50% needed more methanol to achieve a FAME content of >70%. Recently, Fenton's reagent (i.e., a mixture of $FeSO_4$ and H_2O_2) has gained popularity for obtaining higher lipid recovery with lower solvent use. Wu et al. Wu et al. (2010) noted that the hydroxyl radicals (· OH) produced by the reaction between H_2O_2 and Fe^{2+} ions ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$) may attack specific zones of the microbial cell wall constituted by organic compounds. Eventually, the cell wall undergoes partial degradation, leading to the release of the intracellular materials, as well as lipids, to the bulk of the solution. Consequently, Fenton's techniques

achieved comparatively a higher lipid recovery of 16.8% than the ultrasound and solvent extraction. This method is considered an efficient technique to extract higher lipid by using less solvent as well as less time; however, the lipids transferred in solution can be quickly oxidized by the hydroxyl radicals because of the high residual concentration of Fenton's reactants present in the solution thereby leading to pronounced lipid degradation (Steriti et al., 2014).

Table 5.2 Performance of lipid extraction using different cell disruption methods.

No.	Cell disruption methods used	Organisms used	Lipid content (%)	Time (min)	Reference
1	Dialyzed p1MAN5C solution	*R. toruloides*	11.90	120	(Jin et al., 2012)
2	Microwaves	*C. vulgaris*	11.00	5	(Lee et al., 2010)
3	$H_2O_2 + FeSO_4$	*C. vulgaris*	17.34	3	(Steriti et al., 2014)
4	Ultrasound	*R. toruloides*	11.66	30	(Meullemiestre et al., 2017b)
5	Osmotic shock	*Scenedesmus sp.*	8.00	2880	(Lee et al., 2010)
6	Chloroform and methanol (2:1)	*Schizochytrium* sp. S31	22.00	-	(Byreddy et al., 2015)
7	Monothermosonication	*R. toruloides*	26.94	30	(Meullemiestre et al., 2017b)
8	Electroporation	*L. starkeyi*	31.88	10	This Study

The lipid extraction performance of several methods is compared in Table 5.2 with the results from this study. The comparison shows the better performance (31.88%) of the EP technique over others. Although, Meullemiestre et al. (2017b) achieved quite higher lipid content (26.94%) compare to our study, it can be noted that higher temperature (55 °C) and pressure (2 bars) were employed in their study. In addition, the process was time intensive and required 30 min to achieve maximum lipid content (Meullemiestre et al., 2017b) which is three times higher than the present study. In another report (Lee et al., 2010), only 5 min of treatment time was required to extract maximum lipid (11%) using microwave oven from microalgae (*Chlorella vulgaris*). Though the lipid was extracted within shorter time, the lipid yield was about 3 times

lower than EP. Moreover, the method is less feasible due to high temperature (100 °C) and more energy (2450 MHz) consumption.

Table 5.3 Comparison of microbial cell disruption techniques in terms of energy consumption and environmental impact.

Cell disruption techniques	**Solvent (mL/100 g wet biomass)**	**Experimental conditions (temperature-extraction conditions)**	**Carbon emission (kg CO_2/kg microbial oil extracted)**	**Specific energy consumption (kWh/kg microbial oil extracted)**	**References**
Ultrasound	500.00	20 °C for 30 min	45	0.07	(Günerken et al., 2015; Meullemiestre et al., 2016; Meullemiestre et al., 2017a)
Chloroform: Methanol (2:1)	333.33	20 °C for 30 min	156	175	(Meullemiestre et al., 2016; Meullemiestre et al., 2017a)
Fenton's reagent	100 × 10*	25 °C for 5 min	Not studied	Not studied	(Steriti et al., 2014)
Bead milling	333.33	20 °C for 30 min	28	32	(Doucha & Lívanský, 2008; Meullemiestre et al., 2016)
Homogenizer	Not studied	Not studied	Not studied	0.25	(de Boer et al., 2012)
Microwave	500.00	110 °C for 30 min	265	298	(Meullemiestre et al., 2016)
Electroporation	N/A#	Not studied	Not studied	0.06	(de Boer et al., 2012)
Electroporation	N/A#	27 ± 2 °C for 10 min	Not studied	Not studied	This study

* To stop the disruption reaction, the entire reacting mixture was diluted by adding ten times of ethanol of its original volume; # Not applicable, since there is no individual lipid extraction stage, rather EP was used for direct transesterification.

In terms of environmental impact and energy consumption, the advantages of EP over some other existing technologies is compared in Table 5.3. The comparison suggests EP may have less negative-environmental impact. The cell disruption and lipid extraction by some other conventional (Solvent/Ultrasound/Fenton's reagent) methods entail the evaporation of enormous amounts of solvent resulting in high-energy consumption. Moreover, the solvents introduce toxicity, which is objectionable for industrial application (de Boer et al., 2012). Furthermore, these methods have a high-energy burden associated with either the removal of water or the severe reaction conditions required to process microbial biomass with a high-water content. Therefore, using EP to extract lipid from wet biomass may be a promising and energy-efficient alternative processing route because it is performed at ambient temperatures and does not introduce additional impurities into the process. The process and conditions used also minimize undesirable changes in the target material (Xu et al., 2011).

5.5 Lipid composition and characterization

To verify whether the EP treatment might have affected the quality of the extracted lipids after disruption, direct transesterification was carried out to analyze the composition of lipid in terms of FAME. The lipid composition profile (Figure 5.5) reveals that *L. starkeyi* accumulated mainly long-chain fatty acids with 11 to 18 carbon atoms. The predominant fatty acids were palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1). Lee et al. (2010) stated that the palmitic, stearic, oleic, and linolenic acids are the most common fatty acids contained in biodiesel. In their study, oleic acid (C18:1) and linoleic acid (C18:2) were commonly dominant. Louhasakul et al. (2016) reported similar fatty acid content when they cultured yeast species *Y. lipolytica*. They found oleic acid (C18:1) as the most dominant fatty acid followed by linoleic acid (C18:2) and palmitic acid (C16:0). In another study, similar pattern was observed with the fatty acids produced by *Mortierella isabellina*, where oleic acid (C18:1) was predominant followed by palmitic acid (C16:0) (Harde et al., 2016). The identical lipid profiles was observed in this study compared with other literatures, which suggest that lipid composition might not be changed or altered after EP treatment.

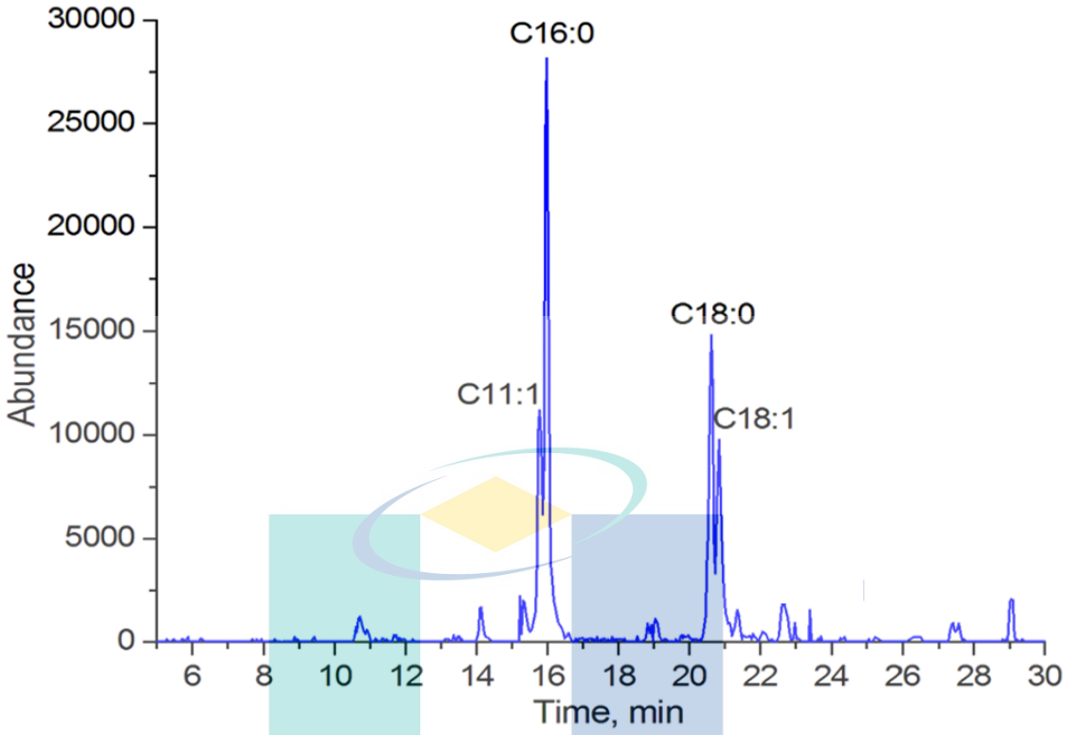

Figure 5.5 Fatty acid methyl esters profile of lipid extracted after EP treatment, accumulated by *L. starkeyi*.

The overall findings of this chapter that the EP is an efficient technique for lipid extraction from microbial biomass. The forces of the pulsing electric field caused significant damage to the cell wall of *L. starkeyi* and the disruption of microbial cells. The extent of cell inactivation was up to 95% when the electrodes were placed at the distance of 2 cm, which provided high treatment intensity (36.7 kWh/m^3). At this condition, maximum lipid (63 mg/g) was extracted when the biomass was treated for 10 min. During the comparison, EP could extract 31.88% lipid while the amount was 11.89% for ultrasonic and 16.8% for Fenton's reagent. The results recommend that the EP is a promising technique for lowering the time and solvent usage for lipid extraction from microbial biomass.

CHAPTER 6

LIPID ACCUMULATION PERFORMANCE OF B. CEREUS, L. STARKEYI, AND THEIR CO-CULTURE THROUGH POME BIOREMEDIATION

6.1 Introduction

In this chapter, the lipid accumulation ability of *B. cereus*, *L. starkeyi*, and by their co-culture was studied by following the bioremediation of POME. A combined approach has been presented to bioremediate POME wastewater and concurrently synthesize microbial lipids using different inoculums. The effect of different POME concentration and different microbes on lipid production were discussed. The lipid accumulation capability was also compared with different inoculums and the quality of the lipids were analyzed by GC-MS.

6.2 Lipid accumulation capacity of *B. cereus* through bioremediation of POME

The prospect of POME wastewater as a medium for bacteria cultivation was not only evaluated by biomass production but also for lipid contents. The lipid accumulation capability of *B. cereus* in different concentrations of POME has been presented in Table 6.1. The highest lipid content was achieved for 50% (v/v) POME, followed by 25%, 75%, 100% POME. The lowest amount of biomass as well as lipid productivity and lipid content were obtained for 100% (undiluted) POME. Generally, high initial organic load is responsible for higher concentration of intermediate metabolites during the biomass growth that leads to the lower lipid yield and COD removal (Cristiani-Urbina et al., 2000). In contrast, the maximum amounts of lipid attained from 50% POME might be due to the consistent presence of the C/N ratio as mentioned above. Usually, the lipid accumulation of a microorganism through wastewater assimilation is greatly influenced by its biomass growth and C/N ratio (Yousuf et al., 2010).

Table 6.1 Lipid accumulation of *B. cereus* in different concentrations of POME medium.

Substrate (POME)	Dry biomass (g/L)	Lipid (g/L)	Lipid content (wt.%, dry weight basis)	Lipid productivity (g/L/day)
25%	6.86 ± 0.21	0.91 ± 0.10	13.26 ± 0.68	0.15
50%	8.09 ± 0.36	1.46 ± 0.05	18.04 ± 0.97	0.24
75%	6.97 ± 0.24	0.82 ± 0.09	11.76 ± 0.46	0.13
100%	5.01 ± 0.16	0.27 ± 0.07	5.38 ± 0.41	0.04

The result of lipid accumulation and productivity for different POME samples (Table 6.1) can be correlated with the growth profiles (Chapter 4; Figure 4.1) of *B. cereus*, in terms of similar effects of the dilution factor. Interestingly, the higher lipid accumulation was found for 25% POME rather than for 75% POME concentration, although the higher biomass production was observed for 75% POME. A similar phenomenon was reported by Cheah et al. (2018b), where the biomass growth was not correlated to the lipid accumulation since the energy is usually diverted to cell growth rather than to lipid accumulation in a nutrient rich condition. It is worth noting that the high lipid content does not associate with the biomass productivity (Qi et al., 2016). For instance, nitrogen possesses a positive role in biomass growth but not in accumulating lipids. In general, the lipid accumulation starts after the nutrients are used up for biomass growth. In a previous study, it was already proved that the cultivation strategy such as nitrogen starvation can bring significant improvement in lipid production (Chen et al., 2015). The lipid accumulation by *Chlorella* sp. accumulated higher lipids when it was stimulated by nitrogen-deficient conditions (Qi et al., 2016). It can be postulated from the cultivation cycle of *B. cereus* that the bacteria started to grow very rapidly after the adaptation period (Chapter 4; Figure 4.1) by duplicating the cells using organics and nutrients in the exponential phase, leading to the higher biomass production. While most of the nutrients were utilized, the cells then reached the stress condition due to a lack of nutrients and organics.

There are some other studies describing the use of POME and other wastewater as a substrate to produce microbial lipids, and their results are summarized in Table 6.2. Although the growth of *B. cereus* biomass was relatively quite high in this study compared to others with various bacterial strains, which is reviewed in Table 6.2, the lipid content was quite low in most cases. This might be due to the different culture conditions and substrate characteristics.

Table 6.2 Microbial lipid harvesting performance of several bacterial strains from different wastewater.

Microorganisms	**Culture conditions**	**Biomass (g/L)**	**Lipid (g/L)**	**Lipid content (wt.%)**	**References**
Pseudomonas sp.	POME (30% v/v), 3 day	1.91 ± 0.07	0.31 ± 0.04	16.04	(Zhang et al., 2016)
R. opacus DSM 43205	Dairy waste, 4 days	3.71	1.89	51.00	(Kumar et al., 2015)
B. subtilis HB1310	Cotton stalk hydrolysate, 2 days	5.70	2.30	39.80	(Zhang et al., 2014b)
R. opacus	Dairy wastewater	4.00	3.16	79.00	(Gupta et al., 2018)
B. subtilis HB1310	Cotton stalk hydrolysate	5.70	2.27	39.82	(Zhang et al., 2014b)
Gordonia sp. DG	Orange waste	0.12	0.06	50.00	(Gouda et al., 2008)
B. cereus (own isolate)	POME (50%, v/v)	8.09 ± 0.36	1.46 ± 0.05	18.04 ± 0.97	Present study

The cell disruption during the lipid extraction process was visualized under the FESEM, as presented in Figure 6.1. It is clearly seen in the FESEM image that EP damaged the microbial cells by creating cleavages on the cell surface during the lipid extraction process. Similar findings were observed in a previous study, where the higher treatment intensity induced irreversible permeabilization of the cell wall leading to its disruption by triggering pore formation (Sheng et al., 2011). EP treatment produced a less resistant barrier towards the intrusion of organic solvent, hence the lipid extraction was enhanced. Thus, the EP technique led to enhanced cell disruption and, subsequently, to a

higher lipid yield in the medium. According to our study, EP demonstrated better lipid extraction efficiency than the ultrasound, Fenton's reagent, or solvent extraction. It was already discussed in previous chapter (Chapter 5) that EP could be a potential technique for lowering the time and solvent usage for lipid extraction from microbial biomass. The lipid accumulation efficiency of *B. cereus* has been compared with other typical bacterial strains, namely *R. opacus* ATCC 51881 and *P. aeruginosa* ATCC 15442, as presented in Figure 6.2.

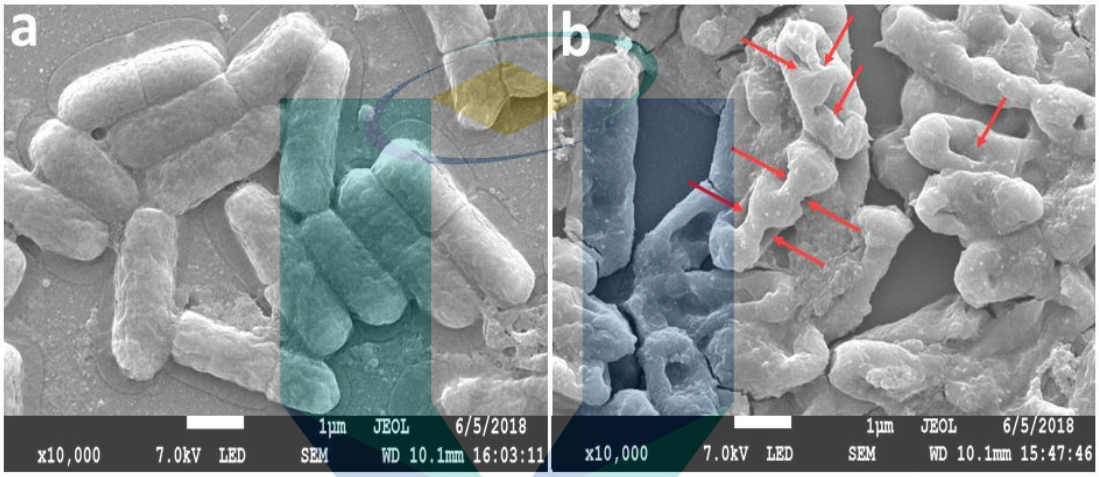

Figure 6.1 The visualization of *B. cereus* cell by FESEM (a) before and (b) after EP treatment (red arrows indicate the cleavage of cells).

It was observed that the *B. cereus* showed a higher biomass harvesting capacity as well as lipid accumulation, although the lipid content was higher in a commercial strain of *R. opacus*. This result revealed that the wild type bacterial strain isolated from wastewater could be more efficient in biomass and lipid accumulation from POME when compared to the commercial strains. This might be due to the greater tolerance of wild type bacterial isolate to the adverse environmental conditions of wastewater (Bala et al., 2015).

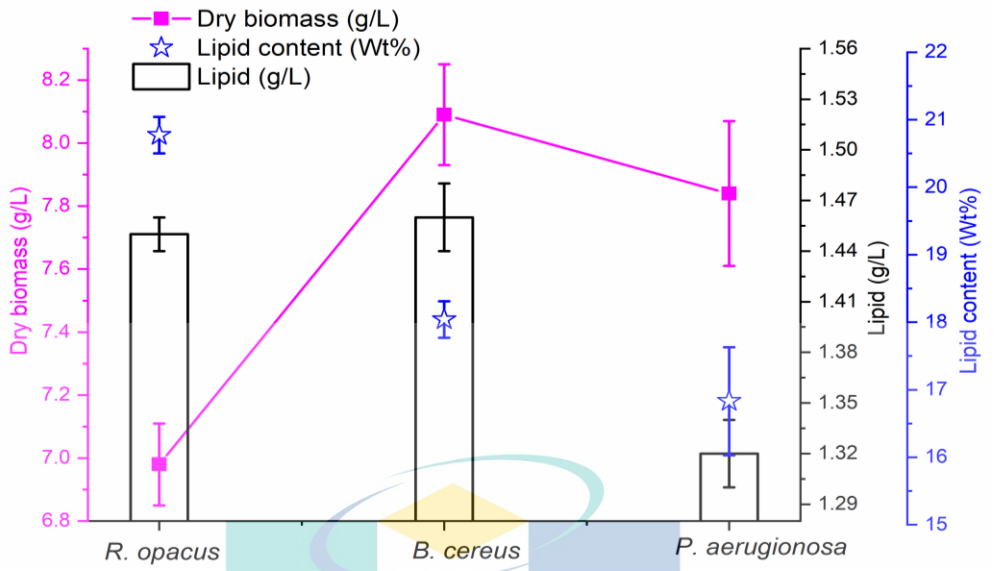

Figure 6.2 The lipid accumulation capacity of several strains of bacteria cultured in 50% POME.

The lipid content accumulated by *B. cereus* was converted to FAME using a direct transesterification reaction. *B. cereus* can accumulate long chain fatty acids with 11 to 18 carbon atoms (Figure 6.3). Among the different fatty acids, undecanoic acid (C11:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) were observed as the predominant fatty acids. Lee et al. (2010) reported that the linoleic, oleic, palmitic, and stearic acids are the most common fatty acids contained in biodiesel; hence, the lipids produced by *B. cereus* could be considered as a potential alternative to plant oils. In addition, Cea et al. (2015) reported that the lipid profile of *Bacillus sp.* generally contains a low degree of unsaturated long chain fatty acids (such as C18:1) and has the highest neutral lipid contents; therefore, the lipid products of these bacteria could be a promising raw material for biodiesel production. Finally, it can be concluded that the *B. cereus* cultivated in a low cost POME medium can accumulate lipids, which demonstrates its ideal biodiesel production properties and the similarity of its fatty acid composition to that of plant oils; thus, these fatty acids could potentially be used for producing high quality biodiesel.

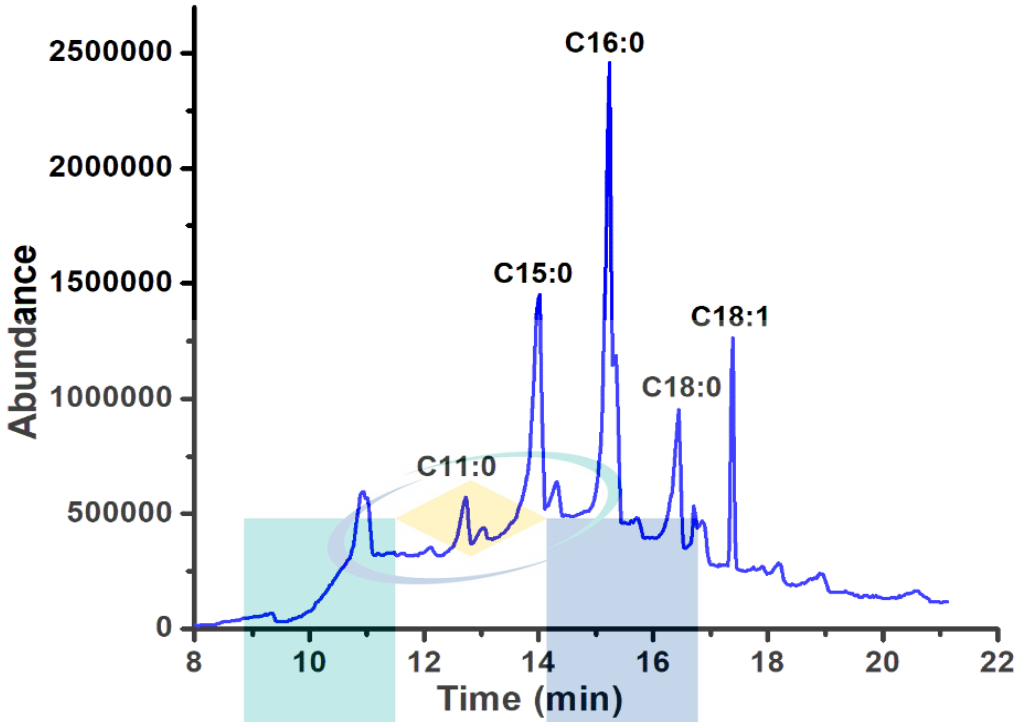

Figure 6.3 Composition of lipid in terms of Fatty acid methyl esters, accumulated by *B. cereus* in POME.

6.3 Lipid accumulation of *L. starkeyi* through the biodegradation processes

The lipid contents of *L. starkeyi* cultured in POME are reported in Table 6.3. The experimental data show that the highest amount (21.29%) of intracellular lipids was obtained using the 50% dilution of POME. However, further dilution of POME (25%) did not show any significant enhancement. The minimum levels of biomass wt., lipid content and lipid yield were observed when using undiluted (100%) POME due to the higher amounts of inhibitors.

Table 6.3 Lipid accumulation of *L. starkeyi*, cultured in different concentrations of POME.

POME sample	Dry biomass (g/L)	Lipid (g/L)	Lipid content (wt.%, dry weight basis)	Lipid productivity (g/L/day)
25%	6.60 ± 0.18	1.02 ± 0.11	15.45 ± 0.33	0.20
50%	7.61 ± 0.23	1.62 ± 0.08	21.29 ± 0.82	0.32
75%	6.73 ± 0.21	0.94 ± 0.10	13.97 ± 0.30	0.19
100%	4.30 ± 0.11	0.32 ± 0.06	7.44 ± 0.25	0.06

These results can be correlated with the growth profiles (Chapter 4, Figure 4.4) of *L. starkeyi*, showing similar effects of the dilution factor. Lipid accumulation in *L. starkeyi* is influenced by its growth and the C/N ratio (Yousuf et al., 2010). The higher amounts of lipid extracted from the 50% POME might be due to the cogent presence of the C/N ratio as described above. Louhasakul et al. (2016) found a lipid concentration of 1.15 g/L for two-fold diluted POME using *Yarrowia lipolytica*. They were able to increase the lipid concentration up to 1.64 g/L for undiluted POME by adding a commercial N-source such as ammonium sulphate. However, in the present study, a lipid concentration of up to 1.62 g/L was obtained without the addition of any external or commercial nutrient sources. There are some other studies describing the use of POME as a substrate to produce microbial lipids, and their results are summarized in Table 6.4.

Although the growth of *L. starkeyi* biomass was relatively low in this study compared to others with various yeast strains, which is reviewed in Table 6.4, the lipid content was quite high in most cases. The literature suggests that the accumulation of microbial lipids depends on cultivation conditions (Yousuf et al., 2010), as well as the physiology and the production of secondary metabolites (Beopoulos et al., 2009). This could explain the difference in lipid accumulation results from the present study.

Moreover, lipid accumulation in yeasts and molds is affected by nitrogen and carbon sources, C/N ratio, temperature, agitation, and pH of the medium (Back et al., 2016). However, some of these factors (C/N ratio, temperature and agitation) were not considered in this study and will be addressed in future studies to maximize lipid accumulation.

Table 6.4 Performance of oleaginous yeasts to produce microbial lipid from POME.

Microorganisms	Culture conditions	Biomass (g/L)	Lipid (g/L)	Lipid content (wt%)	References
Y. lipolytica	Two-fold diluted effluent	3.79 ± 0.04	1.15 ± 0.11	30.34 ± 1.45	(Louhasakul et al., 2016)
Y. lipolytica	un-diluted effluent +nitrogen source	5.68 ± 0.32	1.64 ± 0.03	28.87 ± 0.26	(Louhasakul et al., 2016)
Candida silvae	POME (90%v/v)	11.71 ± 0.8	1.85	15.81 ± 1.9	(Marjakangas et al., 2015)
Galactomyces geotrichum	POME (90%v/v)	10.92 ± 0.5	0.81	7.42 ± 1.4	(Marjakangas et al., 2015)
Lecythophora hoffmannii	POME (90%v/v)	13.01 ± 0.5	1.22	9.42 ± 1.3	(Marjakangas et al., 2015)
Graphium penicillioides	POME (90%v/v)	12.91 ± 0.1	2.37	18.41 ± 1.0	(Marjakangas et al., 2015)
L. starkeyi	POME (50% v/v)	7.61 ± 0.23	1.62 ± 0.08	21.29 ± 0.82	Present study

It is important to note that the carbon substrate feedstock constitutes a major part of the production cost in the accumulation of microbial lipids, (Srinophakun et al., 2017). Moreover, in accordance with green energy, closed-loop system and zero-waste utilization principles, industrial waste should be exploited to produce value added products (Srinophakun et al., 2017) because the bioconversion of such wastes to lipids could reduce lipid production cost and, simultaneously, rid the environment of highly polluted wastes (Arous et al., 2016). Therefore, a wide variety of low-cost raw materials such as olive oil mill wastewater (Yousuf et al., 2010), agro industrial wastewater (Arous et al., 2016), winery wastewater (Salgado et al., 2016), and dairy industry wastewater (Pirozzi et al., 2013) have gained renewed attention as feedstock for microbial lipid production. The results of the present study suggest that POME can be a promising feedstock for large scale microbial lipid production.

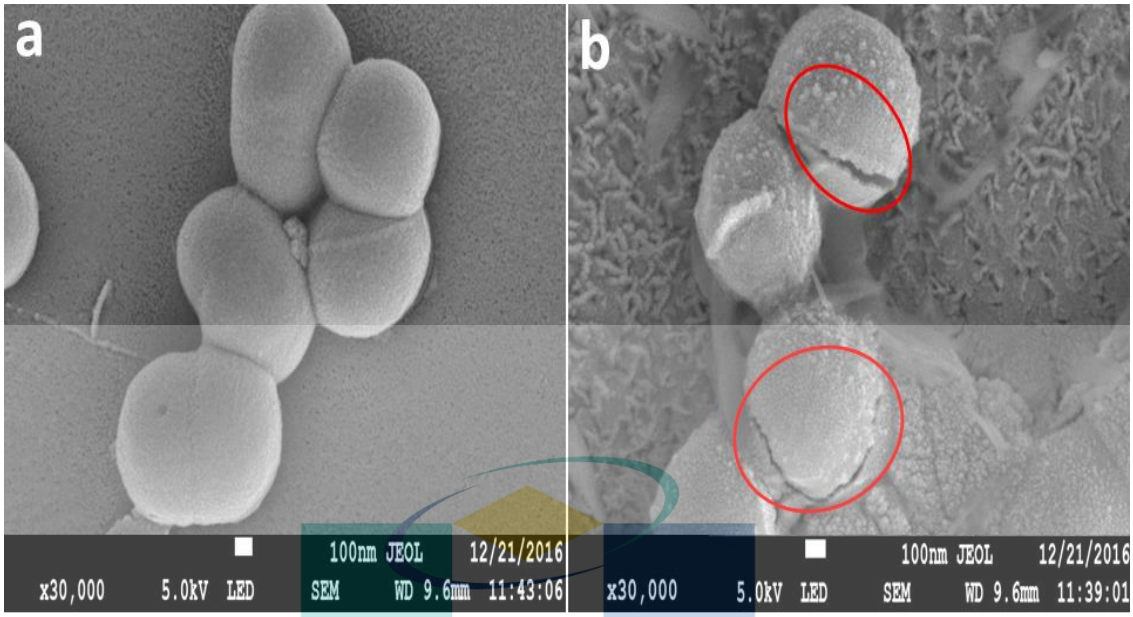

Figure 6.4 The visualization of *L. starkeyi* cell by FESEM (a) before and (b) after EP treatment.

The cell disruption during the lipid extraction process was visualized under the FESEM, as presented in Figure 6.4. It is clearly seen in the FESEM image that EP damaged the microbial cells during the lipid extraction process. Direct transesterification was conducted to analyse the composition of lipids in terms of FAME. The lipid-composition profile (Figure 6.5) reveals that *L. starkeyi* mainly accumulated long-chain fatty acids with 16 and 18 carbon atoms. The predominant fatty acids were palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1). Louhasakul et al. (2016) reported similar fatty acid content when they cultured the yeast species *Y. lipolytica* in POME. They found oleic acid (C18:1) as the most dominant fatty acid followed by linoleic acid (C18:2) and palmitic acid (C16:0). Although, Huang et al. (2011) observed the dominance of myristic acid (C14:0) when *L. starkeyi* was cultured in fish meal wastewater, but this accumulation occurred due to the supplementation of glucose and yeast extract. A similar pattern was observed with the fatty acids produced by *Mortierella isabellina*, where oleic acid (C18:1) was the predominant fatty acid followed by palmitic acid (C16:0) (Harde et al., 2016).

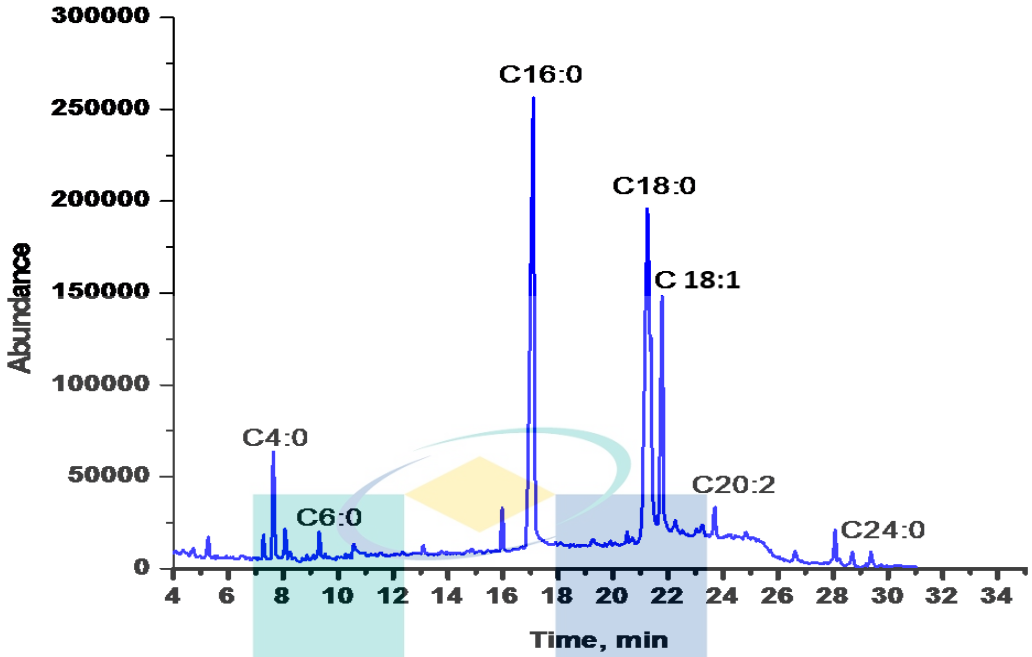

Figure 6.5 Composition of lipid in terms of Fatty acid methyl esters, accumulated by *L. starkeyi* in POME.

Generally, fats and fatty acids from animal and plant sources are unacceptable to some sections of the society due to the conflict of food vs fuel. Therefore, it is quite advantageous that oleaginous microorganisms are capable of producing a wide range of polyunsaturated fatty acids, similar to plant oils, and could reduce pressure to use food sources (Pleissner et al., 2017). In this study, a potential microbial lipid production process is proposed for large scale production and easy recovery of fatty acids and biodiesel synthesis. In addition, the fatty acid profile shows high amounts of saturated fatty acids and mono-unsaturated fatty acids and low values of linolenic acid in the harvested microbial oils, indicating the system's suitability to produce high-quality biodiesel (Tsolcha et al., 2017). Therefore, the results of this study suggest that combining wastewater treatment with microbial biodiesel production can greatly lessen the financial pressure of biodiesel production and reduce environmental hazards.

6.4 Lipid accumulation by the co-culture of *B. cereus* and *L. starkeyi*

The biomass growth and lipid accumulation performance of *B. cereus*, *L. starkeyi* and their co-culture were studied in the 50% POME during 144 h of treatment and is presented in Figure 6.6. It is interesting to note that the highest biomass and lipid

accumulation were obtained for co-culture inoculum than the monocultures. In the monocultures, *B. cereus* achieved a higher biomass growth, but a lower amount of lipids compared to *L. starkeyi*.

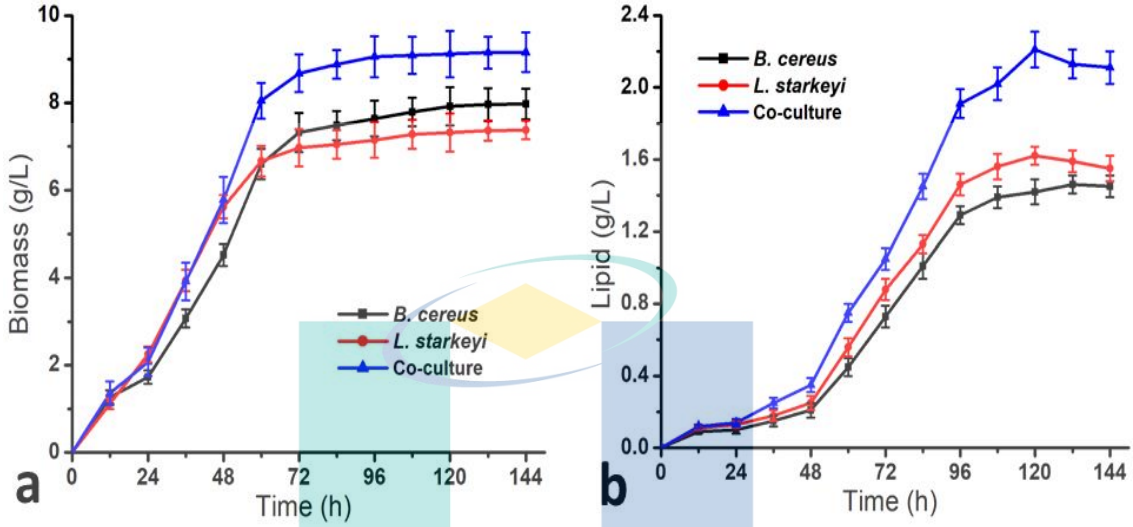

Figure 6.6 (a) Biomass production, and (b) lipid accumulation performance by different inoculums during 144 h of cultivation in the 50% POME.

As can be seen from Figure 6.6a, the biomass production followed a similar trend for all inoculums (co-culture and monocultures) where they increased drastically during the first 72 h, and thereafter gradually increased until the end. However, they showed some incurving behavior after 96 h which could be interpreted as a tendency to reach a plateau. As depicted in Figure 6.6b, the lipid accumulation was considerably increased with the time. However, three different stages were found in lipid accumulation. In first stage (0-48 h), lipid accumulation was slow, can be called lag phase; lipid accumulation exponentially increased in the second phase (48-96 h); lipid accumulation reached a plateau in the third stage (96-144 h). Lipid accumulation reached a plateau in different time depending on the inoculums. The highest lipid of 1.46 g/L was produced on day 6 by *B. cereus*, whereas *L. starkeyi* produced a maximum lipid of 1.62 g/L on day 5, and then decreased to 1.55 g/L. It was observed that the co-culture inoculum obtained 1.5 times higher lipid accumulation compared to the monocultures. It is apparent that the maximum lipid production of 2.21 g/L was achieved by co-culture inoculum on day 5, thereafter gradually decreased to 2.11 g/L on day 6. The highest biomass and lipid accumulation achieved by *B. cereus*, *L. starkeyi* and co-culture inoculum are presented in Table 6.5.

Table 6.5 Total biomass and lipid accumulation of different inoculum in the 50% POME.

Inoculum	Dry biomass (g/L)	Lipid (g/L)	Lipid content (wt.%, dry weight basis)	Lipid productivity (g/L/day)
B. cereus	8.09 ± 0.36	1.46 ± 0.05	18.04 ± 0.97	0.24
L. starkeyi	7.61 ± 0.23	1.62 ± 0.08	21.29 ± 0.82	0.32
Co-culture	9.16 ± 0.41	2.21 ± 0.10	24.12 ± 0.93	0.44

The co-culture of *B. cereus* and *L. starkeyi* accumulated higher lipid compared to the single cultures could be ascribed to the synergistic interaction between *B. cereus* and *L. starkeyi* which substantially enhanced the growth and biomass production by the co-culture (Cai et al., 2007). The synergistic interactions between these two microbes could have enhance their metabolic abilities hence a higher biomass growth was achieved by the co-culture than that of monocultures (El-Masry et al., 2004). Furthermore, the co-culture inoculum would have reduced carbon and nitrogen earlier than monocultures, and subsequently, such nitrogen limitation facilitated faster lipid accumulation (Cheah et al., 2018c; Chen et al., 2015) by the co-culture.

The lipid accumulation was increased simultaneously with the increase in biomass production as a function of incubation time. However, the rate of lipid accumulation was slow during the first 48 h because the microorganisms utilize their metabolic energy to grow in nutrition rich condition rather that lipid accumulation. After day 4, the cell growth was observed to decrease and there was no significant enhancement in the biomass production. This might be resulted from the depletion of the nutrients in the culture medium. However, lipid accumulation was increased significantly at the same time, and reached a plateau on day 5. It is worth noting here that the lipid accumulation was higher and faster for the co-culture than the pure cultures as biomass concentration was higher. A decrease in lipid production after day 5, could be due to the degradation of storage lipids by microbes. This fact is well established that the oleaginous microorganisms usually seen to reserve lipids during the growth and nitrogen starvation phase and degrade it under carbon starvation conditions (Cheirsilp et al., 2011; Fakas et

al., 2007; Karim et al., 2019). In conclusion we could declare that, this mixed culture strategy led to significant improvements in growth and biomass concentration, and consequently, higher lipid production was achieved because the mixed culture inoculum effectively utilized a broad range of compounds from the POME wastewater and contributing to creation of carbon and nitrogen depletion stage earlier than monocultures, and subsequently, nitrogen limitation triggers the lipid accumulation (Cheah et al., 2018c; Chen et al., 2015).

Table 6.6 Performance of co-culture to produce microbial lipids from wastewater.

Strains	**Co-culture consortia**	**Substrates**	**Biomass (g/L)**	**Lipid (g/L)**	**Lipid content (wt.%)**	**References**
Pseudomonas sp. on *C. sorokiniana* CY-1	Microalgae-bacteria, 1:1	30% (v/v) POME, 5 days	2.04	0.33	16.04	(Cheah et al., 2018c)
R. gultinis and *C. vulgaris*	Yeast-microalgae, 1:1	Sugar cane plant wastewater (molasses), 7 days	4.63	2.88	-	(Cheirsilp et al., 2011)
Scenedesmus obliquus with *Pseudomonas* sp.	Microalgae-bacteria, 2:1	BG11 medium, 10 days	2.96	0.68	21.10	(Wang et al., 2015a)
Rhizobium radiobacter and *C. vulgaris*	Bacteria-microalgae, 0.3	BG11 medium, 12 days	-	-	25.80	(Wang et al., 2015b)
B. cereus and *L. starkeyi*	Bacteria-yeast, 1:1	50% POME, 5 days	9.16	2.21	24.12	Present study

The lipid accumulation performance of this study has been compared with some other co-cultures and is presented in Table 6.6. As displayed in Table 6.6, the efficiency of the co-culture (in the present study) is comparable with different co-cultures reported by some recent studies. The higher lipid accumulation the two species used in this study might have symbiotic mutualism (Cai et al., 2007). Therefore, yeast and bacteria co-culture could be considered as effective inoculum for microbial lipid accumulation. However, Cheirsilp et al. (2011) achieved comparatively higher lipid accumulation (2.88 mg/L) using yeast and microalgae co-culture and sugar cane plant wastewater (molasses) as feed. This might be due to the higher concentration of sugars (contained 62% of total

sugar) in the substrate. In contrast, POME is a complex substrate, that was used in our study without addition of any commercial nutrients. Moreover, the result reported in their study was achieved in an optimized condition.

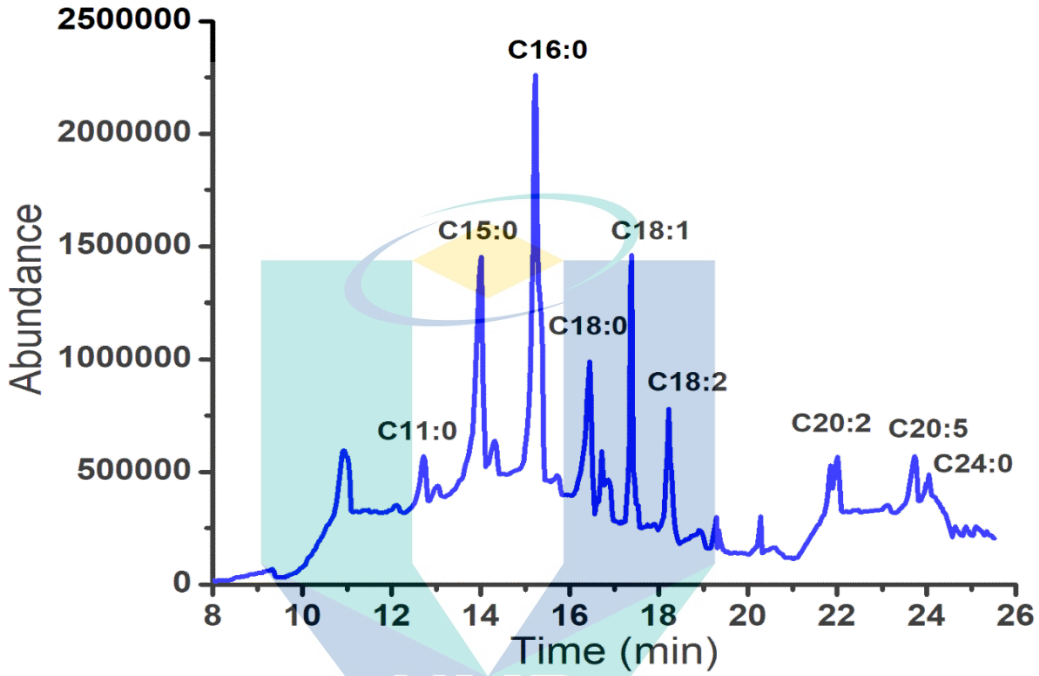

Figure 6.7 Composition of lipid in terms of Fatty acid methyl esters, accumulated by a co-culture of *B. cereus* and *L. starkeyi* in POME.

The lipid accumulated by *B. cereus* and *L. starkeyi* was converted to FAME, and the long chain fatty acids, namely undecanoic acid (C11:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2), were found to be predominant which indicates their potential as a biodiesel feedstock (Figure 6.7). It can be observed that the majority of fatty acids obtained in the co-culture were C16 and C18, which have been identified as best suited for biofuel production (Ojo et al., 2015). Biolipid produced by co-culture cultivated in POME are then suitable biodiesel candidate as it has great biodiesel properties with high percentages of C16:0 and C18:1 obtained in the study. Higher percentage of C16 increases heating value and cetane number, meanwhile C18 exhibits higher oxidative stability with better storage capacity (Nur, 2014). Furthermore, C18:1 and C18:2 helps in mixing of fuel and air, reducing ignition delay period and thus improve combustion efficiency (Zhang et al., 2018). Nevertheless, some polyunsaturated fatty acids (such as C20:2, C20:5) were obtained from the co-culture, which indicated its potential towards the production of other

bioproducts (Rodolfi et al., 2017). The economic feasibility could be further improved through biorefinery of expensive bioproducts like polyhydroxyalkanoates (PHA), polyhydroxy butyrate (PHB), pharmaceuticals, byproducts recycling, reuse of glycerol produced after transesterification (Cheah et al., 2018a) or incorporation of biogas production from residual lipid extracted biomass (Sapci & Morken, 2014).

In conclusion, the co-culture of a bacteria and yeast was evaluated as an effective inoculum in terms of its intracellular lipid accumulation capability. The co-culture inoculum achieved significantly higher lipid accumulation (2.21 g/L) with a higher productivity (0.44 g/L/day) than that of monocultures. In monocultures, the yeast strain *L. starkeyi* was more efficient for lipid production than the bacterial strain *B. cereus*. On the other hand, *B. cereus* accumulated a significantly higher biomass (8.09 g/L) and lipid content (1.46 g/L) than the other bacterial strains, such as *R. opacus* and *P. aeruginosa*. The overall findings of this research work suggest that the co-culture inoculum could be a potential inoculum for enhancing lipid accumulation through the bioremediation of POME. Additional engineering design, research and optimization studies are required to enhance biomass production rates to levels sufficient for economic and sustainable lipid production through the remediation of POME and pursue both environmental resilience and an eco-friendly method for biofuel production.

CHAPTER 7

OPTIMIZATION OF YEAST AND BACTERIA CO-CULTURE FOR LIPID ACCUMULATION AND BIOREMEDIATION OF POME USING RESPONSE SURFACE METHODOLOGY

7.1 Introduction

In the present chapter, the optimization of some operational conditions such as pH, temperature, and time with co-culture inoculum compositions was performed using CCD to maximize the lipid production as well COD removal efficiency. The statistical model used to predict the performance of lipid accumulation and COD removal efficiency of the co-culture of *B. cereus* and *L. starkeyi* using RSM is discussed in this section. The data was analysed using Design Expert (Version-7.1.6). The functions were optimized for the lipid production performance and COD removal efficiency.

7.2 Development of regression model analysis

Under the experimental conditions of CCD, several experiments were conducted with a different combination of parameters to study the interactive effects of independent variables (Figures 7.1, A5 and Tables 7.1, 7.2, A2). The COD removal efficiency and lipid accumulation data were fitted using a quadratic model. The predicted data and experimental data were very close to each other and distributed symmetrically, indicating that the good agreement between actual and predicted data for both COD removal efficiency and lipid accumulation. This result ascribed that the applied models were acceptable to predict the responses (the COD removal and lipid accumulation efficiency).

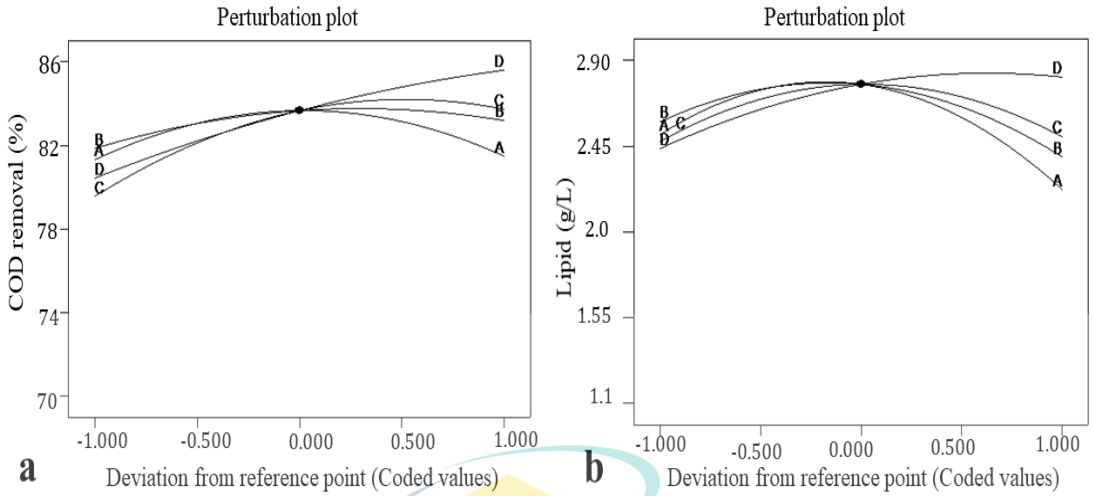

Figure 7.1 Perturbation plot illustrating the interactions of the independent variables using different combination of parameters for a) COD removal efficiency and b) lipid accumulation. Where, A, B, C, and D are representing the inoculum compositions, medium pH, incubation temperature, and cultivation time, respectively.

The interactive effect of inoculum composition, medium pH, incubation temperature, and cultivation time, on the COD removal and lipid accumulation performance over operation period are illustrated in Figure 7.1. The effect of responses can be observed by the variations in the variables that are varied away from the single reference point when one variable remains constant. The perturbation plot Figure 7.1b showed that, inoculum composition, medium pH and incubation temperature have significant impact on lipid production, where the effects of all the variables at the center point in the design space are compared.

Table 7.1 Variables used in the fermentation of POME for COD removal and lipid accumulation.

Variables	**Levels**				
	-2	**-1**	**0**	**+1**	**+2**
Concentration of inoculum A (%), x_1	10	30	50	70	90
pH, x_2	5.5	6.0	6.5	7.0	7.5
Temperature (°C), x_3	27.5	30.0	32.5	35.0	37.5
Incubation time (h), x_4	70	80	90	100	110

Similar profile was observed in case of COD removal, (Figure 7.1a), a steep curvature in inoculum composition, 'A' curve, suggests that COD removal is sensitive to this factor. The comparatively slightly-flat; B' and 'C' curve shows slight lower sensitive than 'A' to COD removal. It is clear from the perturbation plot that inoculum composition has comparatively more significant factor to influence lipid yield as well as COD removal. The independent variables used for the COD removal and lipid accumulation are showed in Table 7.1. It was observed that the COD removal efficiency and lipid accumulation data were fitted using a quadratic model. The high R^2 values (close to 1) for the model suggesting the experimental data were adjusted with the model (Ghafari et al., 2009).

Table 7.2 Central composite design (coded values) to maximize the COD removal and lipid accumulation using a co-culture inoculum.

Run No.	x_1	x_2	x_3	x_4	y_1	y_2
1	0.000	0.000	0.000	0.000	85	2.81
2	-1.000	1.000	-1.000	-1.000	73	2.05
3	0.000	0.000	2.000	0.000	80	1.97
4	0.000	-2.000	0.000	0.000	74	1.76
5	2.000	0.000	0.000	0.000	75	1.13
6	-1.000	1.000	-1.000	1.000	78	2.18
7	0.000	0.000	0.000	0.000	83	2.77
8	-1.000	-1.000	-1.000	1.000	81	2.25
9	1.000	1.000	1.000	1.000	82	1.58
10	1.000	1.000	1.000	-1.000	75	1.14
11	-1.000	-1.000	-1.000	-1.000	73	1.67
12	-1.000	-1.000	1.000	-1.000	76	1.99
13	-1.000	1.000	1.000	-1.000	80	1.14
14	0.000	2.000	0.000	0.000	83	1.57
15	0.000	0.000	0.000	0.000	84	2.8
16	1.000	-1.000	1.000	-1.000	80	1.58
17	1.000	-1.000	1.000	1.000	82	1.78
18	-1.000	1.000	1.000	1.000	82	1.48
19	1.000	-1.000	-1.000	-1.000	73	1.25
20	1.000	-1.000	-1.000	1.000	78	1.55
21	-1.000	-1.000	1.000	1.000	81	1.76
22	0.000	0.000	0.000	0.000	82	2.71
23	0.000	0.000	0.000	0.000	83	2.76
24	-2.000	0.000	0.000	0.000	73	1.25
25	0.000	0.000	0.000	-2.000	75	1.56
26	0.000	0.000	0.000	2.000	86	2.89
27	1.000	1.000	-1.000	1.000	79	1.25
28	0.000	0.000	-2.000	0.000	70	1.37
29	0.000	0.000	0.000	0.000	85	2.8
30	1.000	1.000	-1.000	-1.000	73	1.16

x_1=Concentration of inoculum A (%); x_2=pH; x_3=Temperature (°C); x_4=Incubation time (h); y_1=COD removal (%); y_2=Lipid accumulation (g/L).

The empirical relationship between responses (COD removal efficiency and lipid accumulation) and the coded variables were analyzed using the equations given below, Equation 7.1, and Equation 7.2.

$$\text{COD removal efficiency (Coded)} = 83.67 + 0.083 \times x_1 + 0.67 \times x_2 + 2.08 \times x_3 + 2.58 \times x_4 - 0.37 \times x_1 \times x_2 + 0.13 \times x_1 \times x_3 + 0.000 \times x_1 \times x_4 + 0.13 \times x_2 \times x_3 + 0.000 \times x_2 \times x_4 - 0.50 \times x_3 \times x_4 - 2.27 \times x_1^2 - 1.15 \times x_2^2 - 2.02 \times x_3^2 - 0.65 \times x_4^2 \quad (7.1)$$

$$\text{Lipid accumulation (Coded)} = 2.78 - 0.14 \times x_1 - 0.093 \times x_2 + 0.012 \times x_3 + 0.19 \times x_4 - 0.13 \times x_1 \times x_2 + 0.17 \times x_1 \times x_3 + 0.013 \times x_1 \times x_4 - 0.11 \times x_2 \times x_3 + 9.375E - 003 \times x_2 \times x_4 - 0.022 \times x_3 \times x_4 - 0.41 \times x_1^2 - 0.29 \times x_2^2 - 0.29 \times x_3^2 - 0.15 \times x_4^2 \quad (7.2)$$

The experimental and predicted values for COD removal efficiency and lipid accumulation are presented in Figure 7.2. As apparent from Figure 7.2, the predicted data and experimental data were very close to each other and distributed symmetrically, which indicating that the good agreement between actual and predicted data for both the COD removal efficiency and lipid accumulation (Figures 7.2a-b). This result ascribed that the applied models were acceptable to predict the responses (the COD removal and lipid accumulation efficiency).

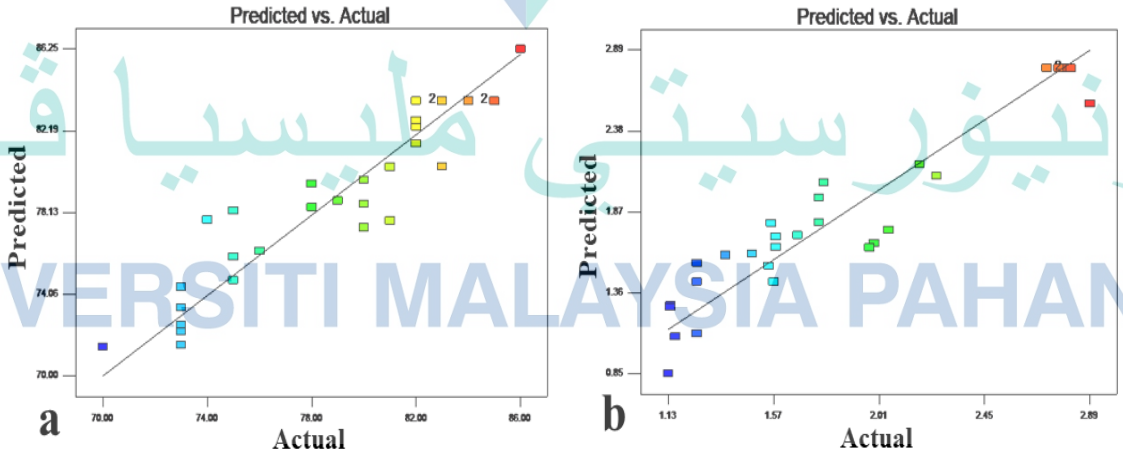

Figure 7.2 Maximum performance between predicted vs actual values (a) COD removal efficiency, (b) lipid accumulation.

7.3 Statistical analysis

The ANOVA table of models for the COD removal efficiency and lipid accumulation are shown in Table 7.2. The F-values of the x and y axes were determined from the model in the case of COD removal efficiency (7.85) and lipid accumulation (10.28), which ascribes that the models are substantially accurate and significant. Moreover, the adj-R^2 values were observed as 0.7678 and 0.8175 for COD removal efficiency and lipid accumulation, respectively. The higher values of adj-R^2 indicate that the model was developed using adequate data hence the prediction of model was reliable to obtain accurate performance. Here it can be noted that the pred-R^2 values for COD removal efficiency and lipid accumulation were observed as 0.3622 and 0.4591, respectively. In addition, the higher adeqaute precision values of 9.693 and 10.604 for COD removal efficiency and lipid accumulation respectively, indicate an adequate signal and therefore, this model can be used to navigate the design space. Nevertheless, the high R^2 values (close to 1) for the model suggesting the experimental data were adjusted with the model. The higher R^2 values for COD removal efficiency (R^2, 0.8799) and lipid accumulation (R^2, 0.9056) confirming the model successfully explained the relationship between variable parameters and operational conditions accurately. The R^2 values for COD removal efficiency lipid accumulation indicate that 87.99% and 90.56% of the variability in response could be explained by these fitted models.

7.4 Chemical oxygen demand removal efficiency

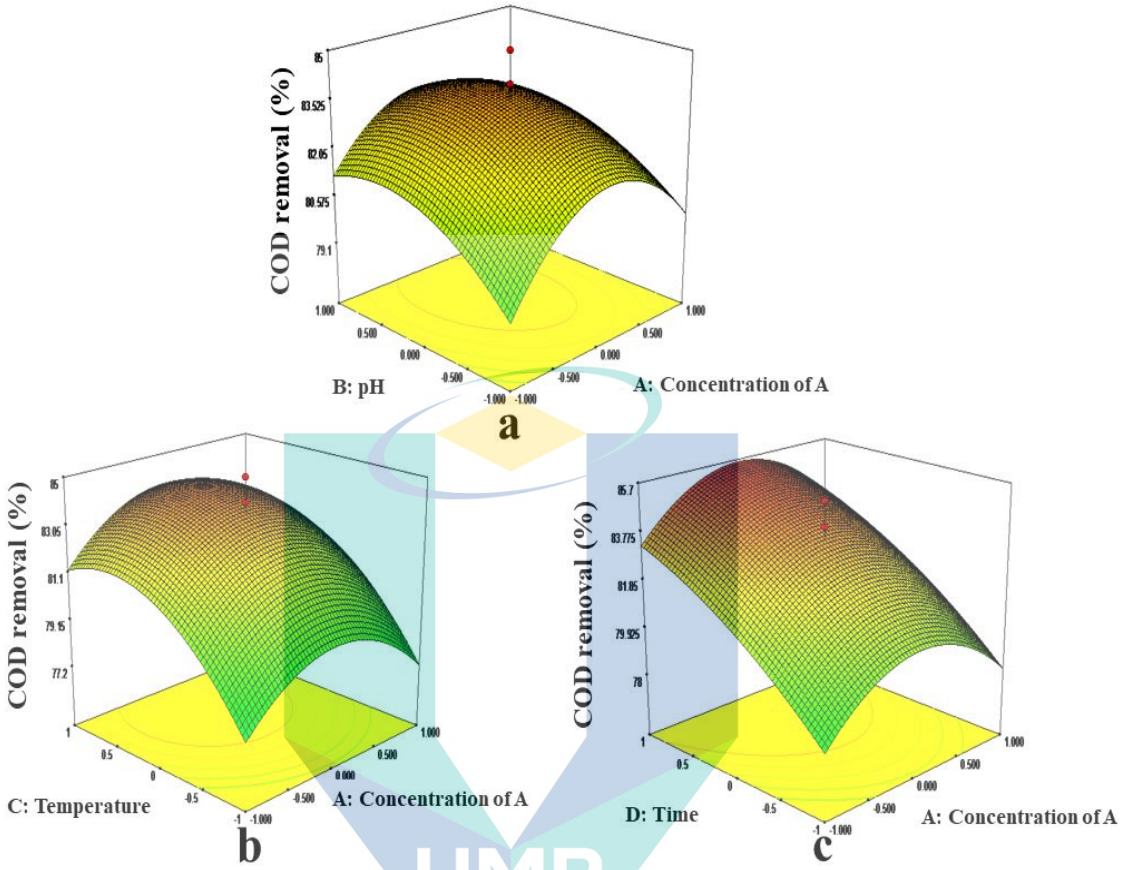

Figure 7.3 Three-dimensional response surface plots showing the relationship between (a) pH and inoculum composition, (b) temperature and inoculum composition, (c) cultivation time and inoculum composition.

The effect of targeted operational parameters such as inoculum composition, pH, temperature, and time on the COD removal efficiency are presented in Figures 7.3a-c and 7.4a-c. As can be seen from the figures, the COD removal efficiency increased from pH of 6.0 to 6.5 and the highest COD removal was obtained at pH ~6.5. Likewise, the COD removal efficiency was improved by increasing the temperature from 30.0 ℃ to 32.5 ℃, and the maximum COD removal efficiency was achieved at 32.5 ℃. Nevertheless, further increasing in the pH and temperature substantially reduced the COD removal efficiency. Besides, the inoculum composition also significantly influences on the COD removal efficiency. The COD removal efficiency enhanced with the increasing concentration of inoculum A (*B. cereus*) from 30% to 50%, then started to decrease and further increased to 70%. Interestingly, the equal ratio of both microbes obtained highest COD removal efficiency. On the other hand, the COD removal efficiency enhanced with the function of cultivation time and reached the plateau after 90 h of operation.

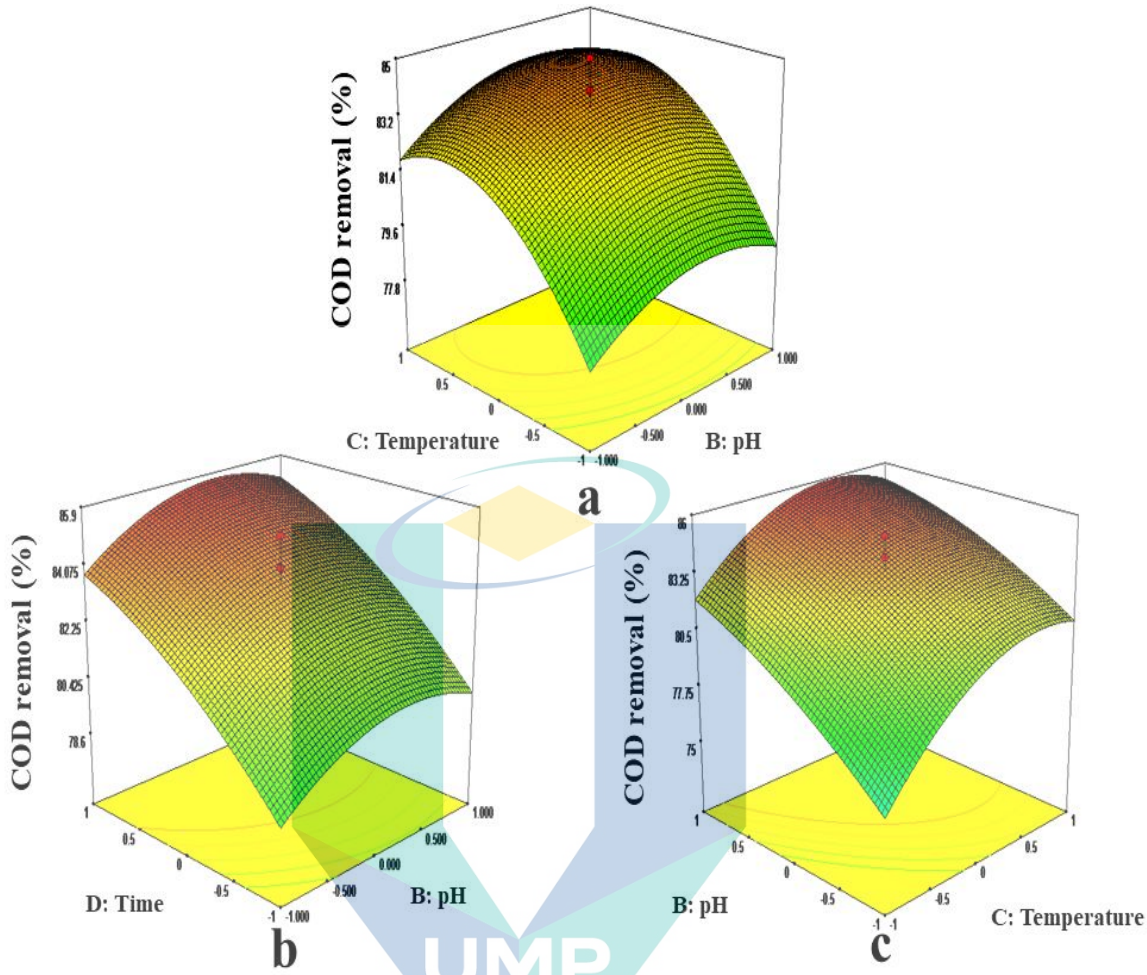

Figure 7.4 Three-dimensional response surface plots showing the relationship between (a) temperature and pH, (b) cultivation time and pH, (c) cultivation time and temperature.

ANOVA analysis for quadric model of COD removal efficiency is presented in Table 7.3. The P values of the co-efficients of the model indicate the significance ($P < 0.05$). The P value of the model (0.0001) showed the model was very significant. The linear term temperature (x_3: 0.0003), time (x_4: < 0.0001) were also significant whereas the quadratic effect of inoculum composition (x_1^2: <0.0001), pH (x_2^2: 0.0142), and temperature (x_3^2: 0.0002) were found to be very significant. However, the interactions of the four manipulated variables were not significant to COD removal efficiency.

Table 7.3 Analysis of variance (ANOVA) for quadric model of COD removal efficiency.

Source	Sum of Squares	Degree of freedom (DF)	Mean Square	F-Value	p-value Prob > F	Clarification
Model	514.55	14	36.75	7.85	0.0001	significant
x1-Concentration of A	0.17	1	0.17	0.036	0.8529	
x2-pH	10.67	1	10.67	2.28	0.1520	
x3-Temperature	104.17	1	104.17	22.24	0.0003	
x4-Time	160.17	1	160.17	34.20	< 0.0001	
x1x2	2.25	1	2.25	0.48	0.4988	
x1x3	0.25	1	0.25	0.053	0.8204	
x1x4	0.000	1	0.000	0.000	1.0000	
x2x3	0.25	1	0.25	0.053	0.8204	
x2x4	0.000	1	0.000	0.000	1.0000	
x3x4	4.00	1	4.00	0.85	0.3700	
x1^2	141.44	1	141.44	30.20	< 0.0001	
x2^2	36.01	1	36.01	7.69	0.0142	
x3^2	112.01	1	112.01	23.92	0.0002	
x4^2	11.44	1	11.44	2.44	0.1389	
Residual	70.25	15	4.68			
Lack of Fit	62.92	10	6.29	4.29	0.0608	not significant
Pure Error	7.33	5	1.47			
Core Total	584.80	29				

7.5 Lipid accumulation capacity

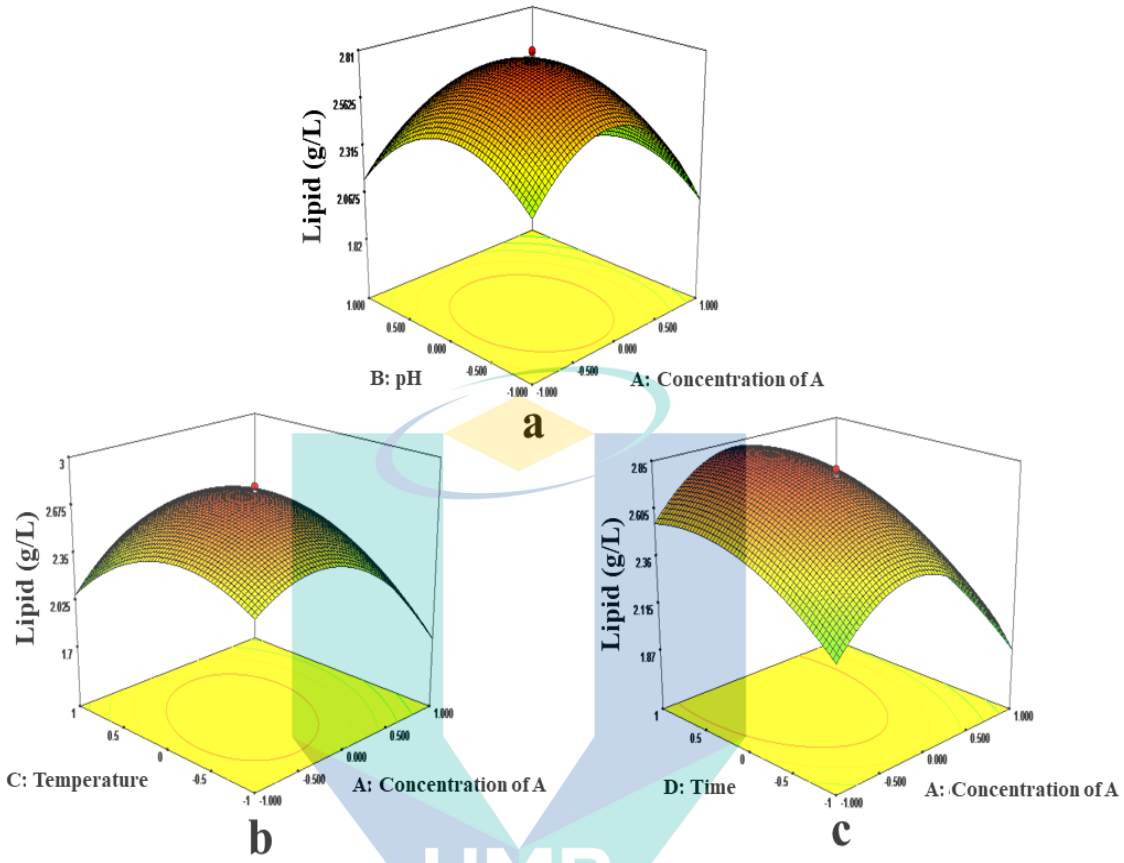

Figure 7.5 Three-dimensional response surface plots showing the relationship between (a) pH and inoculum composition, (b) temperature and inoculum composition, (c) cultivation time and inoculum composition.

The interaction between dependent and independent parameters has been analysed with the 3D response curves. The lipid accumulation was varied by modifying operational parameters (i.e., inoculum composition, pH, temperature, and time) as presented in Figures 7.5a-c and 7.6a-c. It is apparently seen that the inoculum composition, pH, and incubation time significantly influenced lipid accumulation. The equivalent concentration (50:50) of yeast and bacteria seems to be an optimum ratio for obtaining maximum lipid production. Besides, the lipid accumulation increased with the augmenting temperature, but the temperature above 32.5 ℃ showed the descending trend. The lipid accumulation increased when raising the pH from 6.0 to 6.5; and the maximum lipid accumulation was obtained at pH ~6.5. However, further increment in pH negatively affected on the upward trend of lipid accumulation. Likewise, the incubation time maintained a positive correlation on lipid accumulation while the maximum lipid

accumulation was reached after 90 h of operation; but subsequently the lipid accumulation showed a quasi-steady fashion.

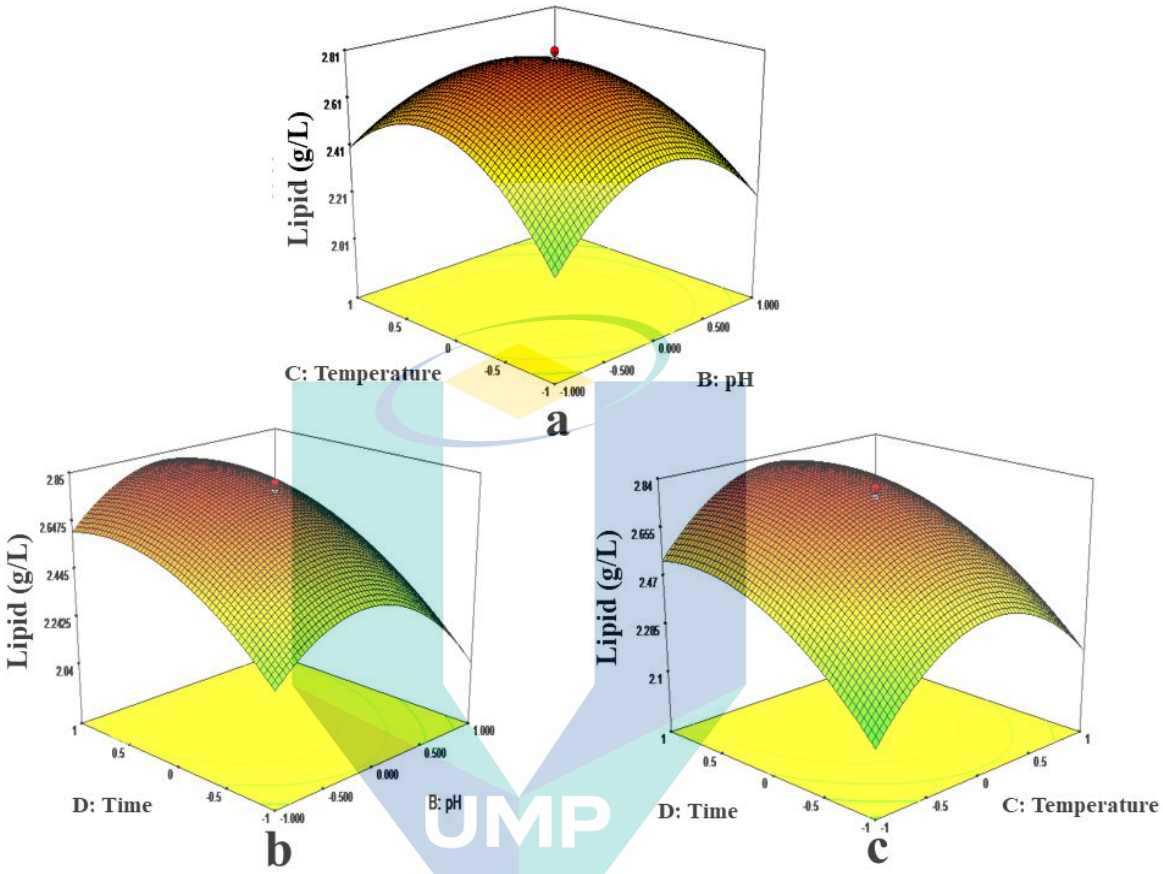

Figure 7.6 Three-dimensional response surface plots showing the relationship between (a) temperature and pH, (b) cultivation time and pH, (c) cultivation time and temperature.

ANOVA analysis for quadric model of lipid accumulation performance is presented in Table 7.4. The P values of the co-efficients of the model indicate the significance ($P < 0.05$). The P value of the model (< 0.0001) showed the model was very significant. The linear term inoculum composition (x_1: 0.0146), pH (x_2: 0.0423), and time (x_4: 0.0027) were also significant whereas the quadratic effect of all four variables such as inoculum composition (x_1^2: <0.0001), pH (x_2^2: < 0.0001), and temperature (x_3^2: < 0.0001), and time (x_4^2: 0.0079) were found to be very significant. Moreover, On assessing interactions between the manipulated experimental variables, the P values of cross-terms provide some insights, as they do regarding effects of each single variable by itself. The interactions of the inoculum composition and temperature (x_1x_3: 0.0208) was significant to lipid accumulation performance.

Table 7.4 Analysis of variance (ANOVA) for quadric model of lipid accumulation.

Source	Sum of Squares	Degree of freedom (DF)	Mean Square	F-Value	p-value Prob > F	Clarification
Model	9.47	14	0.68	10.28	< 0.0001	significant
x1-Concentration of A	0.50	1	0.50	7.62	0.0146	
x2-pH	0.21	1	0.21	3.15	0.0423	
x3-Temperature	3.504E-003	1	3.504E-003	0.053	0.8206	
x4-Time	0.85	1	0.85	12.87	0.0027	
x1x2	2.756E-003	1	2.756E-003	0.042	0.8406	
x1x3	0.44	1	0.44	6.67	0.0208	
x1x4	2.756E-003	1	2.756E-003	0.042	0.8406	
x2x3	0.18	1	0.18	2.71	0.1204	
x2x4	1.406E-003	1	1.406E-003	0.021	0.8857	
x3x4	7.656E-003	1	7.656E-003	0.12	0.7378	
x1^2	4.58	1	4.58	69.58	< 0.0001	
x2^2	2.31	1	2.31	35.02	< 0.0001	
x3^2	2.29	1	2.29	34.72	< 0.0001	
x4^2	0.62	1	0.62	9.36	0.0079	
Residual	0.99	15	0.066			
Lack of Fit	0.98	10	0.098	70.54	< 0.0001	significant
Pure Error	6.950E-003	5	1.390E-003			
Core Total	10.46	29				

Based on mathematical equations, individual parameters were optimized to get optimum performance of COD removal efficiency and lipid accumulation. The optimum parameters are shown in Table 7.5. According to Table 7.5, the maximum performance can be achieved while the inoculum composition, pH, temperature, and incubation time would be 50:50, 6.50, 32.5 ℃ and 90 h, respectively. Under these conditions, the model estimated that the maximum lipid accumulation efficiency would be 2.95 g/L while the maximum COD removal efficiency would be 86.54%. To justify the model prediction, an experiment was conducted in three replicates by following model given operational parameters. At optimized process parameters, we observed that the COD removal efficiency and lipid accumulation were 84.57% and 2.81 g/L respectively, with error

values (2.28 and 4.75, respectively) between predicted and experimental results. The validation confirmed a good agreement between predicted responses and experimental results. Hence, this model could be applied to predict the performance of co-culture inoculated reactor with varying operational conditions.

Table 7.5 The best operational conditions for the process and experimental results to confirm optimization capability.

Factors				Desirability	Response [a]					
					COD removal (%)			Lipid accumulation (g/L)		
x_1 (%)	x_2	x_3 (°C)	x_4 (h)	0.928	Prediction	Actual	Error (%) [b]	Prediction	Actual	Error (%) [b]
50	6.50	32.50	90		86.54	84.57 ± 2.35	2.28	2.95	2.81 ± 0.32	4.75

a= Observed response value: mean± S.D. (n=3), b = [difference between predicted value and actual value/Predicted value] × 100

7.6 Discussion

The effect of operational parameters such as pH, inoculum composition, time, and temperature on the performance of lipid accumulation and wastewater treatment (COD removal) were optimized using RSM. Recently, the co-culture inoculums have gained attention from the researchers for enhancing lipid production because the collective output of co-culture is usually higher than that of the monoculture systems (Islam et al., 2018a; Kim et al., 2016; Venkataraman et al., 2011). In the present study, different ratios of both microbes were investigated to have highest performance in the lipid accumulation and COD removal efficiency. However, among them, the equivalent concentration (50:50) of both microbes achieved the highest performance compared to other ratios. This might be due to the synergistic interaction between two microbes. Indeed, the ratio of inoculum greatly influences the microbial synergistic interaction as well as lipid accumulation. For instance, the higher biomass (2.04 g/L) and productivity (185.71 mg/L/d) were attained by co-cultivation of *C. sorokiniana* CY-1 and *Pseudomonas* sp. with a ratio of 1:1 in the POME. At this inoculum ratio, lipid content (16.04%) was about two fold higher than other ratios of 2:1 or 1:2. Similarly, Cheirsilp et al. (2011) obtained higher lipid production (2.88 ± 0.16 g/L) and COD removal (79.0 ± 1.1%) using a co-culture of microalga *C. vulgaris* and yeast *R. glutinis* in the ratio of 1:1 (Cheah et al., 2018c). In another study, the ratio of 0.20–0.25 for bacteria and microalgae was observed as optimum culture conditions to obtain maximum lipid accumulation (Wang et al.,

2015b). Therefore, it can be said that the inoculum composition significantly influences on the microbial growth and lipid accumulation.

The initial pH of the fermentation medium is an important environmental factor for biomass formation as well as lipid production. This is because the initial medium pH found to have significant effects on the cell growth and products formation (Abdelhamid et al., 2019; Shoaib et al., 2018). It has been reported that the H^+ concentration severely influences the growth and sporulation process of microbes (Lilly & Barnett, 1951). Besides, the external pH of the medium may affect the plasma membrane permeability; consequently, the change of the external pH affects the membrane osmosis towards the absorption of the different ions and nutrients from the surrounding medium (Shoaib et al., 2018). The effect of medium pH on the performance of lipid accumulation has already been reported in many studies (Abdelhamid et al., 2019; Cheirsilp et al., 2011; Shoaib et al., 2018). Generally, the microbes require a pH close to neutral for their optimal growth. In our study, it was observed that lipid accumulation improved with the increase in initial pH from 5.5 to 6.5, and then slowly decreased for further enhancement to 7.5. This could be due to the smaller biomass growth in increased medium pH. Because the consumption or breakdown of phenolic compounds of POME by *L. starkeyi* could leads to the formation of hydroxide ions (Islam et al., 2018c), and pH was increased as a function of the fermentation time. Consequently, the pH of the medium would have been reached to a basic condition faster and inhibited the microbial growth. In some other studies, it was shown that the acidity was also playing an inhibitory role in lipid accumulation as it negatively influences the microbial cell growth as well as the metabolism (Sadabad & Gholikandi, 2017). Zhao et al. (2017) showed that the maximum growth of *Lactobacillus* bacteria was at an initial pH of 6.5, however, pH below 5.0 was not favorable for the growth of bacteria. In the case of yeast, the growth was maximum at pH 6.0, but similar growth was observed at pH 5.5 (Zhao et al., 2017). Therefore, it is concluded that the neutral pH is imperative to achieve optimal microbial growth as well higher COD removal and lipid accumulation.

Nevertheless, the incubation temperature significantly influenced on the biomass formation and lipid accumulation. The results of several previous studies indicated that growth of biomass as well as lipid accumulation was enhanced with increasing incubation temperature from 25 °C to 35 °C (Ali et al., 2017; Shoaib et al., 2018). This might be due

to the optimum growth temperature of (Abdelhamid et al., 2019). We found that the COD removal efficiency and lipid production increases with augumenting the incubation temperature from 27.5 ℃ to 32.5 ℃, and gradually decrease at incubation temperatures higher than 32.5 °C. Our results were in accordance with Zhao et al. (2017) that the optimum temperatures for both bacteria and yeast were observed at 28-32 ℃, and they obtained the highest growth rate at 31°C using a co-culture of yeast and bacteria. In a recent study by Subhash and Mohan (2014), a temperature of 30 ℃ was found to be suitable for growth and lipid accumulation by oleaginous fungus *A. awamori*. The maximum lipid production (39.0 ± 1.43% lipid/dry biomass) was observed at 30 °C for *P. brevicompactum* NRC 829, while a lower biomass and lipid production was observed at increasing or decreasing incubation temperature (Ali et al., 2017).

The incubation time had a great impact on the performance of biomass growth and lipid accumulation as well as COD removal. The fact that the COD removal efficiency drastically increased in the initial period of operation might be due to the faster growth of microbes. But, after a few days (3 to 4 days), the growth of microbes reached the stationary phase therefore the COD removal efficiency become quasi constant. The COD removal remained almost unchanged after four days, attributed to the fact that the growth of microorganisms was hindered due to the depletion of the nutrients in the culture medium. This is indicating that the consumable carbon source present in the effluent was limited, namely not all of COD could be consumed by the yeast and bacteria. Lipid production also raises with increases of incubation time in the initial period of operation, but it started decreasing after a few days of operation due to the degradation of storage lipids by microbes. This fact is well established that the oleaginous microorganisms usually seen to store lipids at initial stage especially during the lag/log and nitrogen limiting phase, and break it down under carbon limiting conditions (Cheirsilp et al., 2011; Fakas et al., 2007; Karim et al., 2019). In our study, we observed that the lipid accumulation and COD removal was enhanced with increase in time from 80 h to 100 h and obtained a maximum COD removal efficiency (~85%) and lipid accumulation (2.81 g/L) at 90 h of incubation time. These results are congruent with Ali et al. (2017), where they reported that *Aspergillus* spp. obtained maximum lipid production after five days (120 h) of incubation. Likewise, a mixed culture of microalga *C. vulgaris* and oleaginous yeast *R. glutinis* showed highest biomass as well as lipid production after five days of cultivation in industrial waste, then slightly decreased in lipid production on day 7

(Cheirsilp et al., 2011). Ali and El-Ghonemy (2014) noticed that the *Aspergillus* sp. and *Trichoderma viride* NRC314 achieved maximum lipid production after five days incubation. Therefore, the COD removal efficiency and lipid accumulation were significantly dependent on the incubation time.

To sum up, the statistical model suggests that the maximum lipid accumulation of 2.95 g/L and COD removal of 86.54%, could be achieved under the conditions of inoculum composition, 1:1; pH 6.5; temperature, 32.5 ℃; and incubation time, 90 h. An experiment was conducted by following optimum parameters given by the model to justify the accuracy of model predictions. We obtained less than 5% deviation between model predictions and real experiments results which apparently justify the uses of the proposed model. The results of the present study suggest that the performance of microbial lipid production and bioremediation could be improved using yeast bacteria co-culture inoculum in certain optimum conditions. However, further study needs to be conducted to develop a mechanistic model to know the insight of co-culture inoculum influences on lipid production and POME bioremediation.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

Important conclusions based on the objectives are presented below:

Objective 1: To investigate the bioremediation efficiency of POME by *B. cereus*, *L. starkeyi* and their co-culture inoculum.

The potential of POME bioremediation by *B. cereus*, *L. starkeyi* and co-culture inoculum was evaluated by using different POME concentrations. It was observed that the moderately dilute solution of POME (50%) showed higher microbial growth and offered a significantly higher degree of bioremediation. Furthermore, higher remediation was achieved using the co-culture inoculum in the 50% POME, especially COD and BOD, demonstrating removal efficiencies of 83.66% and 77.34%, respectively. Nevertheless, POME treated with co-culture inoculum obtained a higher GI value than the other samples (treated by pure cultures and untreated) due to the significant remediation of detrimental organics present in the POME.

The process technology implications: These results obtained from objective 1 suggest that co-culture of yeast and bacteria is a suitable inoculum to degrade the toxic components of POME and could improve the seed germination rate if treated POME is used for irrigation purposes. Moreover, the aerobically treated POME could be well utilized on an industrial scale for the betterment of agricultural crops with proper dilution. This diluted and treated by co-culture effluent could be used both for invigorating the seeds and for further irrigating crops or nurseries. Therefore, POME bioremediation by *B. cereus*, *L. starkeyi* and their co-culture inoculum would be an attractive option to achieve the environmental resilience.

Objective 2: To study the efficiency of microbial lipid extraction using a novel EP technique compared to several conventional extraction techniques.

This study revealed that EP is a potentially viable technique for disrupting the microbial cell wall, which is a rate limiting factor for the lipid extraction step in biodiesel synthesis. The EP demonstrated a higher lipid extraction efficiency of 31.88% (wt.%) compared to the ultrasound (11.89%), Fenton's reagent (16.80%), and solvent extraction (9.60%).

The process technology implications: The results obtained from objective 2 suggest that the EP technique could be a promising pretreatment techinique to overcome the limitations of the conventional technique such as greater chemical use, high retention time, and high energy input. This is a significant achievement from industrial and environmental point of view. Moreover, the analysis of lipid composition reported in this study suggests that the EP technique does not alter the extracted lipid profile, which is very promising for the direct transesterification of microbial lipid to biodiesel using wet biomass. Thus the steps for biomass drying could be avoided.

Objective 3: To evaluate lipid accumulation performance of *B. cereus*, *L. starkeyi* and their co-culture through POME bioremediation.

The co-culture inoculum was found to have potential for the highest biomass growth (9.16 g/L) and lipid accumulation (2.21 g/L), with a lipid content of 24.12% (dry weight basis) in the 50% (v/v) POME. The co-culture of *B. cereus* and *L. starkeyi* accumulated higher lipid compared to the single cultures because of the synergistic interaction between *B. cereus* and *L. starkeyi* which substantially enhanced the growth and biomass production by the co-culture.

The process technology implications: The results achieved from objective 3 suggest that POME can be a promising feedstock for large scale microbial lipid production instead of using commercial medium like glucose. Generally, fats and fatty acids from animal and plant sources are unacceptable, to some extent, due to the conflict of food vs fuel. Oleaginous microorganisms are capable of producing a wide range of saturated and polyunsaturated fatty acids, similar to plant oils, and could reduce pressure to use food sources.

Objective 4: To optimize the yeast-bacteria co-culture for enhancing lipid production and bioremediation efficiency using response surface methodology (RSM).

The statistical model showed that a maximum lipid accumulation of 2.95 g/L and COD removal of 86.54%, could be achieved under the optimized conditions for inoculum composition, 1:1; pH 6.5; temperature, 32.5 ℃; and incubation time, 90 h. Furthermore, the predicted results were very close to experimental results (<5% deviation), hence the proposed model could be used to predict the lipid accumulation performance of a yeast and bacterium co-culture through the effective COD removal from POME.

The process technology implications: The result of optimization study obtained from objective 4 suggest that the performance of microbial lipid production and bioremediation could be improved using yeast bacteria co-culture inoculum in certain optimum conditions. This finding will pave the way to develop a large-scale production process compatible with industrial development. Therefore, this study provides an alternative way to valorize the POME as a growth media for the production of microbial lipids suitable for biodiesel production and simultaneously provides a strategy to reduce wastewater pollution loads.

8.2 Recommendations

In the present work, the effect of the yeast and bacteria (*B. cereus* and *L. starkeyi*) co-culture was studied in a lab-scale; however, further research is required to evaluate the applicability of co-culture for a large scale POME wastewater remediation. POME was sterilized by autoclaving to inactivate other microbes present in POME as the target of this study was to evaluate the efficiency of several targeted microbes in terms of bioremediation and lipid accumulation. However, autoclaving is not economically feasible on a large-scale thus the next set of experiments should include raw, as-is, in-sterilized POME as the feedstock as this will be the real feedstock in the real world.

In this study, EP treatment was employed with a batch reactor in the lab-scale. Further study is required to engineer the EP circuit and placement of electrodes for a large-scale application that may entail flow between the electrodes. Moreover, a more precise and sophisticated EP circuit could be designed which allow real time monitoring of the voltage, frequency, current, pulse duration, etc.

In the present study, the co-culture of a bacteria and yeast was evaluated as an effective inoculum to increase biomass production and intracellular lipid accumulation

capability. However, the more deeper insights is rquired to reveal the mechanism of synergistic relationships between yeast and bacteria by analyzing extracellular metabolites in the fermentation medium. For example, yeast can produce some organic acids (glycerol, propionic acid, pyruvic and acetic acids), glycidyl ether, and palmitic acid (may be inhibitory, reduce pH) and the contents of some amino acids (glycine and proline) which may be beneficial for lipid accumulation.

The parameters of co-culture inoculum were optimized using RSM modelling tools, but the mechanistic model should be developed using differential equations and mathematical equations in future study. Moreover, only four parameters such as the inoculum composition, pH, temperature, and incubation time were studied here; but more parameters like aeration/shaking conditions, nutrients supplements etc. can be explored in the future study.

REFERENCES

Abdelhamid, S. A., Hussein, A. A., Asker, M. S., El Sayed, O. H., & Mohamed, S. S. (2019). Optimization of culture conditions for biodiesel production from Egyptian isolate *Penicillium commune* NRC2016. *Bulletin of the National Research Centre, 43*(1), 15.

Agarry, S. (2017). Bioelectricity generation and treatment of petroleum refinery effluent by *Bacillus cereus* and *Clostridium butyricum* using microbial fuel cell technology. *Nigerian Journal of Technology, 36*(2), 543-551.

Ageitos, J. M., Vallejo, J. A., Veiga-Crespo, P., & Villa, T. G. (2011). Oily yeasts as oleaginous cell factories. *Applied microbiology and biotechnology, 90*(4), 1219-1227.

Ahmad, A., Bhat, A., Buang, A., Shah, S., & Afzal, M. (2018). Biotechnological application of microalgae for integrated palm oil mill effluent (POME) remediation: a review. *International Journal of Environmental Science and Technology*, 1-26.

Ahmad, A., Buang, A., & Bhat, A. H. (2016). Renewable and sustainable bioenergy production from microalgal co-cultivation with palm oil mill effluent (POME): A review. *Renewable and Sustainable Energy Reviews, 65*, 214-234. doi:10.1016/j.rser.2016.06.084

Ahmad, A., & Chan, C. (2009). Sustainability of palm oil industries: an innovative treatment via membrane technology. *Journal of Applied Sciences, 9*(17), 3074-3079.

Ahmad, A., Shah, S., Othman, M., & Abdullah, M. (2014a). Enhanced palm oil mill effluent treatment and biomethane production by aerobic and anaerobic co-cultivation of *Chlorella sp*. *Canadian Journal of Chemical Engineering, 92*, 1636-1642.

Ahmad, A., Shah, S., Othman, M., & Abdullah, M. (2015). Aerobic and anaerobic co-cultivation of *Nannochloropsis oculata* with oil palm empty fruit bunch for enhanced biomethane production and palm oil mill effluent treatment. *Desalination and Water Treatment, 56*(8), 2055-2065.

Ahmad, A., Shah, S. M. U., Buang, A., Othman, M. F., & Abdullah, M. A. (2014b). *Evaluation of aerobic and anaerobic co-digestion of Tetraselmis suecica and oil palm empty fruit bunches by response surface methodology.* Paper presented at the Advanced Materials Research.

Ahmad, F. B., Zhang, Z., Doherty, W. O., & O'Hara, I. M. (2019). The prospect of microbial oil production and applications from oil palm biomass. *Biochemical Engineering Journal, 143*, 9-23.

Akhbari, A., Kutty, P. K., Chuen, O. C., Ibrahim, S., Akhbari, A., Kutty, P. K., . . . Ibrahim, S. (2019). A study of palm oil mill processing and environmental assessment of palm oil mill effluent treatment. *Environmental Engineering Research, 25*(2), 212-221.

Alhaji, M. H., Sanaullah, K., Lim, S.-F., Khan, A., Hipolito, C. N., Abdullah, M. O., . . . Jamil, T. (2016). Photocatalytic treatment technology for palm oil mill effluent (POME) – A review. *Process Safety and Environmental Protection, 102*, 673-686. doi:http://dx.doi.org/10.1016/j.psep.2016.05.020

Alhattab, M., Kermanshahi-Pour, A., & Brooks, M. S.-L. (2019). Microalgae disruption techniques for product recovery: influence of cell wall composition. *Journal of Applied Phycology, 31*(1), 61-88.

Ali, T. H., El-Gamal, M. S., El-Ghonemy, D. H., Awad, G. E., & Tantawy, A. E. (2017). Improvement of lipid production from an oil-producing filamentous fungus, *Penicillium brevicompactum* NRC 829, through central composite statistical design. *Annals of Microbiology, 67*(9), 601-613.

Ali, T. H., & El-Ghonemy, D. H. (2014). Optimization of culture conditions for the highest lipid production from some oleaginous fungi for biodiesel preparation. *Asian Journal of Applied Sciences (ISSN: 2321–0893), 2*(05).

Almohammed, F., Mhemdi, H., & Vorobiev, E. (2016). Pulsed electric field treatment of sugar beet tails as a sustainable feedstock for bioethanol production. *Applied Energy, 162*, 49-57.

Amanullah, A., McFarlane, C., Emery, A., & Nienow, A. (2001). Scale-down model to simulate spatial pH variations in large-scale bioreactors. *Biotechnology and bioengineering, 73*(5), 390-399.

Angerbauer, C., Siebenhofer, M., Mittelbach, M., & Guebitz, G. (2008). Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. *Bioresource technology, 99*(8), 3051-3056.

Arenas, E., Rodriguez Palacio, M., Juantorena, A., Fernando, S., & Sebastian, P. (2017). Microalgae as a potential source for biodiesel production: techniques, methods, and other challenges. *International Journal of Energy Research, 41*(6), 761-789.

Arous, F., Frikha, F., Triantaphyllidou, I.-E., Aggelis, G., Nasri, M., & Mechichi, T. (2016). Potential utilization of agro-industrial wastewaters for lipid production by the oleaginous yeast *Debaryomyces etchellsii*. *Journal of cleaner production, 133*, 899-909.

Asadi, E., Makhdoumi, A., & Asoodeh, A. (2020). Laccase mediator system obtained from a marine spore exhibits decolorization potential in harsh environmental conditions. *Ecotoxicology and Environmental Safety, 191*, 110184.

Awad, G. E., Esawy, M. A., Salam, W. A., Salama, B. M., Abdelkader, A. F., & El-diwany, A. (2011). Keratinase production by *Bacillus pumilus* GHD in solid-state fermentation using sugar cane bagasse: optimisation of culture conditions using a Box-Behnken experimental design. *Annals of Microbiology, 61*(3), 663-672.

Back, A., Rossignol, T., Krier, F., Nicaud, J.-M., & Dhulster, P. (2016). High-throughput fermentation screening for the yeast *Yarrowia lipolytica* with real-time monitoring of biomass and lipid production. *Microbial Cell Factories, 15*(1), 147.

Bala, J. D., Lalung, J., Al-Gheethi, A. A., Kaizar, H., & Ismail, N. (2018). Reduction of Organic Load and Biodegradation of Palm Oil Mill Effluent by Aerobic Indigenous Mixed Microbial Consortium Isolated from Palm Oil Mill Effluent (POME). *Water Conservation Science and Engineering*, 1-18.

Bala, J. D., Lalung, J., & Ismail, N. (2014). Biodegradation of palm oil mill effluent (POME) by bacterial. *Int J Sci Res Publ, 4*(3), 502-511.

Bala, J. D., Lalung, J., & Ismail, N. (2015). Studies on the reduction of organic load from palm oil mill effluent (POME) by bacterial strains. *International Journal of Recycling of Organic Waste in Agriculture, 4*(1), 1-10.

Band, V. I., Crispell, E. K., Napier, B. A., Herrera, C. M., Tharp, G. K., Vavikolanu, K., . . . Trent, M. S. (2016). Antibiotic failure mediated by a resistant subpopulation in *Enterobacter cloacae*. *Nature microbiology, 1*(6), 16053.

Banerjee, A., & Ghoshal, A. K. (2017). Bioremediation of petroleum wastewater by hyper-phenol tolerant *Bacillus cereus*: Preliminary studies with laboratory-scale batch process. *Bioengineered, 8*(5), 446-450.

Baranitharan, E., Khan, M. R., Yousuf, A., Teo, W. F. A., Tan, G. Y. A., & Cheng, C. K. (2015). Enhanced power generation using controlled inoculum from palm oil mill effluent fed microbial fuel cell. *Fuel, 143*, 72-79.

Beccari, M., Bonemazzi, F., Majone, M., & Riccardi, C. (1996). Interaction between acidogenesis and methanogenesis in the anaerobic treatment of olive oil mill effluents. *Water research, 30*(1), 183-189.

Benka-Coker, M., & Ekundayo, J. (1997). Applicability of evaluating the ability of microbes isolated from an oil spill site to degrade oil. *Environmental Monitoring and Assessment, 45*(3), 259-272.

Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.-L., Molina-Jouve, C., & Nicaud, J.-M. (2009). *Yarrowia lipolytica* as a model for bio-oil production. *Progress in lipid research, 48*(6), 375-387.

Bharte, S., & Desai, K. (2018). Techniques for harvesting, cell disruption and lipid extraction of microalgae for biofuel production. *Biofuels*, 1-21.

Bhumibhamon, O., Koprasertsak, A., & Funthong, S. (2002). Biotreatment of high fat and oil wastewater by lipase producing microorganisms. *Kasetsart J.(Nat. Sci.), 36*, 261-267.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology, 37*(8), 911-917.

Borowitzka, M. A. (2013). High-value products from microalgae—their development and commercialisation. *Journal of Applied Phycology, 25*(3), 743-756.

Brennan, L., & Owende, P. (2010). Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews, 14*(2), 557-577. doi:https://doi.org/10.1016/j.rser.2009.10.009

Brianceau, S., Turk, M., Vitrac, X., & Vorobiev, E. (2015). Combined densification and pulsed electric field treatment for selective polyphenols recovery from fermented grape pomace. *Innovative Food Science & Emerging Technologies, 29*, 2-8.

Brigham, C. J., Kurosawa, K., Rha, C., & Sinskey, A. J. (2011). Bacterial carbon storage to value added products.

Byreddy, A. R., Barrow, C. J., & Puri, M. (2016). Bead milling for lipid recovery from thraustochytrid cells and selective hydrolysis of *Schizochytrium* DT3 oil using lipase. *Bioresource technology, 200*, 464-469.

Byreddy, A. R., Gupta, A., Barrow, C. J., & Puri, M. (2015). Comparison of cell disruption methods for improving lipid extraction from *thraustochytrid* strains. *Marine drugs, 13*(8), 5111-5127.

Cai, S., Hu, C., & Du, S. (2007). Comparisons of growth and biochemical composition between mixed culture of alga and yeast and monocultures. *Journal of bioscience and bioengineering, 104*(5), 391-397.

Carlile, M. J., Watkinson, S. C., & Gooday, G. W. (2001). *The fungi*: Gulf Professional Publishing.

Cea, M., Sangaletti-Gerhard, N., Acuña, P., Fuentes, I., Jorquera, M., Godoy, K., . . . Navia, R. (2015). Screening transesterifiable lipid accumulating bacteria from sewage sludge for biodiesel production. *Biotechnology Reports, 8*, 116-123.

Chaijak, P., Lertworapreecha, M., & Sukkasem, C. (2018). Phenol removal from palm oil mill effluent using Galactomyces reessii termite-associated yeast. *Polish Journal of Environmental Studies, 27*(1), 39-44.

Chan, Y. J., & Chong, M. F. (2019). Palm Oil Mill Effluent (POME) Treatment—Current Technologies, Biogas Capture and Challenges *Green Technologies for the Oil Palm Industry* (pp. 71-92): Springer.

Chatzifragkou, A., Makri, A., Belka, A., Bellou, S., Mavrou, M., Mastoridou, M., . . . Papanikolaou, S. (2011). Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species. *Energy, 36*(2), 1097-1108.

Cheah, W. Y., Show, P. L., Juan, J. C., Chang, J.-S., & Ling, T. C. (2018a). Enhancing biomass and lipid productions of microalgae in palm oil mill effluent using carbon and nutrient supplementation. *Energy conversion and management, 164*, 188-197.

Cheah, W. Y., Show, P. L., Juan, J. C., Chang, J.-S., & Ling, T. C. (2018b). Microalgae cultivation in palm oil mill effluent (POME) for lipid production and pollutants removal. *Energy conversion and management, 174*, 430-438.

Cheah, W. Y., Show, P. L., Juan, J. C., Chang, J.-S., & Ling, T. C. (2018c). Waste to energy: the effects of *Pseudomonas sp.* on *Chlorella sorokiniana* biomass and lipid productions in palm oil mill effluent. *Clean Technologies and Environmental Policy, 20*(9), 2037-2045.

Cheah, W. Y., Show, P. L., Juan, J. C., Chang, J.-S., & Ling, T. C. (2018d). Waste to energy: the effects of Pseudomonas sp. on Chlorella sorokiniana biomass and lipid productions in palm oil mill effluent. *Clean Technologies and Environmental Policy*, 1-9.

Cheirsilp, B., & Louhasakul, Y. (2013). Industrial wastes as a promising renewable source for production of microbial lipid and direct transesterification of the lipid into biodiesel. *Bioresource technology, 142*, 329-337.

Cheirsilp, B., Suwannarat, W., & Niyomdecha, R. (2011). Mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* for lipid production from industrial wastes and its use as biodiesel feedstock. *New Biotechnology, 28*(4), 362-368.

Chemat, F., & Khan, M. K. (2011). Applications of ultrasound in food technology: processing, preservation and extraction. *Ultrasonics sonochemistry, 18*(4), 813-835.

Chen, J., Li, J., Dong, W., Zhang, X., Tyagi, R. D., Drogui, P., & Surampalli, R. Y. (2018). The potential of microalgae in biodiesel production. *Renewable and Sustainable Energy Reviews, 90*, 336-346.

Chen, Y., Cheng, J. J., & Creamer, K. S. (2008). Inhibition of anaerobic digestion process: a review. *Bioresource technology, 99*(10), 4044-4064.

Chen, Y., Tang, X., Kapoore, R. V., Xu, C., & Vaidyanathan, S. (2015). Influence of nutrient status on the accumulation of biomass and lipid in *Nannochloropsis salina* and *Dunaliella salina*. *Energy conversion and management, 106*, 61-72.

Chew, K. W., Yap, J. Y., Show, P. L., Suan, N. H., Juan, J. C., Ling, T. C., . . . Chang, J.-S. (2017). Microalgae biorefinery: high value products perspectives. *Bioresource technology, 229*, 53-62.

Chigusa, K., Hasegawa, T., Yamamoto, N., & Watanabe, Y. (1996). Treatment of wastewater from oil manufacturing plant by yeasts. *Water Science and Technology, 34*(11), 51-58.

Chin, M. J., Poh, P. E., Tey, B. T., Chan, E. S., & Chin, K. L. (2013). Biogas from palm oil mill effluent (POME): Opportunities and challenges from Malaysia's perspective. *Renewable and Sustainable Energy Reviews, 26*, 717-726.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology advances, 25*(3), 294-306.

Choi, H.-J., & Lee, S.-M. (2012). Effects of microalgae on the removal of nutrients from wastewater: various concentrations of *Chlorella vulgaris*. *Environmental Engineering Research, 17*(S1), 3-8.

Choi, H., Jeong, S.-W., & Chung, Y.-j. (2006). Enhanced anaerobic gas production of waste activated sludge pretreated by pulse power technique. *Bioresource technology, 97*(2), 198-203.

Choi, S.-A., Lee, J.-S., Oh, Y.-K., Jeong, M.-J., Kim, S. W., & Park, J.-Y. (2014). Lipid extraction from *Chlorella vulgaris* by molten-salt/ionic-liquid mixtures. *Algal Research, 3*, 44-48.

Choong, Y. Y., Chou, K. W., & Norli, I. (2018). Strategies for improving biogas production of palm oil mill effluent (POME) anaerobic digestion: A critical review. *Renewable and Sustainable Energy Reviews, 82*, 2993-3006.

Chowdhary, P., Raj, A., & Bharagava, R. N. (2018). Environmental pollution and health hazards from distillery wastewater and treatment approaches to combat the environmental threats: a review. *Chemosphere, 194*, 229-246.

Clarke, A., Prescott, T., Khan, A., & Olabi, A. (2010). Causes of breakage and disruption in a homogeniser. *Applied Energy, 87*(12), 3680-3690.

Concas, A., Pisu, M., & Cao, G. (2015). Disruption of microalgal cells for lipid extraction through Fenton reaction: modeling of experiments and remarks on its effect on lipids composition. *Chemical Engineering Journal, 263*, 392-401.

Cooney, M., Young, G., & Nagle, N. (2009). Extraction of bio-oils from microalgae. *Separation & Purification Reviews, 38*(4), 291-325.

Coustets, M., Al-Karablieh, N., Thomsen, C., & Teissié, J. (2013). Flow process for electroextraction of total proteins from microalgae. *The Journal of membrane biology, 246*(10), 751-760.

Crampon, C., Mouahid, A., Toudji, S.-A. A., Lépine, O., & Badens, E. (2013). Influence of pretreatment on supercritical CO_2 extraction from *Nannochloropsis oculata*. *The Journal of Supercritical Fluids, 79*, 337-344.

Cravotto, G., Boffa, L., Mantegna, S., Perego, P., Avogadro, M., & Cintas, P. (2008). Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. *Ultrasonics sonochemistry, 15*(5), 898-902.

Cristiani-Urbina, E., Netzahuatl-Muñoz, A. R., Manriquez-Rojas, F. J., Juárez-Ramírez, C., Ruiz-Ordaz, N., & Galíndez-Mayer, J. (2000). Batch and fed-batch cultures for the treatment of whey with mixed yeast cultures. *Process Biochemistry, 35*(7), 649-657.

Dai, C.-c., Tao, J., Xie, F., Dai, Y.-j., & Zhao, M. (2007). Biodiesel generation from oleaginous yeast *Rhodotorula glutinis* with xylose assimilating capacity. *African Journal of Biotechnology, 6*(18).

Dai, J., Li, X., Li, B., & Wang, L. (2011). *Design and modeling of an underwater energy harvesting system.* Paper presented at the Proceedings of the international congress on analytical sciences (ICAS 2011). Kyoto, Japan.

de Boer, K., Moheimani, N. R., Borowitzka, M. A., & Bahri, P. A. (2012). Extraction and conversion pathways for microalgae to biodiesel: a review focused on energy consumption. *Journal of Applied Phycology, 24*(6), 1681-1698.

De Felice, B., Pontecorvo, G., & Carfagna, M. (1997). Degradation of waste waters from olive oil mills by *Yarrowia lipolytica* ATCC 20255 and *Pseudomonas putida*. *Acta Biotechnologica, 17*(3), 231-239.

de San, A. J. d. J., & Parres, J. B. E. (2014). Process and apparatus for extracting biodiesel from algae: Google Patents.

Ding, G. T., Yaakob, Z., Takriff, M. S., Salihon, J., & Rahaman, M. S. A. (2016). Biomass production and nutrients removal by a newly-isolated microalgal strain *Chlamydomonas sp* in palm oil mill effluent (POME). *international journal of hydrogen energy, 41*(8), 4888-4895.

Dong, T., Knoshaug, E. P., Pienkos, P. T., & Laurens, L. M. (2016). Lipid recovery from wet oleaginous microbial biomass for biofuel production: a critical review. *Applied Energy, 177*, 879-895.

Doucha, J., & Lívanský, K. (2008). Influence of processing parameters on disintegration of *Chlorella* cells in various types of homogenizers. *Applied microbiology and biotechnology, 81*(3), 431.

Drees, K. P., Abbaszadegan, M., & Maier, R. M. (2003). Comparative electrochemical inactivation of bacteria and bacteriophage. *Water research, 37*(10), 2291-2300.

Đurđica, K., Davor, K., Slavko, R., Daria, J., Robert, S., & Marina, T. (2019). Electroporation of harvest residues for enhanced biogas production in anaerobic co-digestion with dairy cow manure. *Bioresource technology, 274*, 215-224.

Edwards, D. J., & Truong, D. H. (2011). A comparison of designs for one-step screening and response surface estimation. *Quality and Reliability Engineering International, 27*(8), 1009-1024.

Ehimen, E., Sun, Z., & Carrington, C. (2010). Variables affecting the in situ transesterification of microalgae lipids. *Fuel, 89*(3), 677-684.

El-Fadaly, H., El-Ahmady, N., & Marvan, E. (2009). Single cell oil production by an oleaginous yeast strain in a low cost cultivation medium. *Research Journal of Microbiology, 4*(8), 301-313.

El-Masry, M. H., El-Bestawy, E., & Nawal, I. (2004). Bioremediation of vegetable oil and grease from polluted wastewater using a sand biofilm system. *World Journal of Microbiology and Biotechnology, 20*(6), 551-557.

Enamala, M. K., Enamala, S., Chavali, M., Donepudi, J., Yadavalli, R., Kolapalli, B., . . . Kuppam, C. (2018). Production of biofuels from microalgae-A review on cultivation, harvesting, lipid extraction, and numerous applications of microalgae. *Renewable and Sustainable Energy Reviews, 94*, 49-68.

Enshaeieh, M., Abdoli, A., & Madani, M. (2018). Single cell oil (SCO) production by *Rhodotorula mucilaginosa* and its environmental benefits.

Enshaeieh, M., Abdoli, A., Nahvi, I., & Madani, M. (2012). Bioconversion of different carbon sources in to microbial oil and biodiesel using oleaginous yeasts. *Journal of biology and today's world, 1*(2), 82-92.

Enshaeieh, M., Abdoli, A., Nahvi, I., & Madani, M. (2013). Selection and optimization of single cell oil production from *Rodotorula* 110 using environmental waste as substrate. *Journal of cell and molecular research, 4*(2), 68-75.

Evans, C. T., & Ratledge, C. (1984). Phosphofructokinase and the regulation of the flux of carbon from glucose to lipid in the oleaginous yeast *Rhodosporidium toruloides*. *Microbiology, 130*(12), 3251-3264.

Fakas, S., Galiotou-Panayotou, M., Papanikolaou, S., Komaitis, M., & Aggelis, G. (2007). Compositional shifts in lipid fractions during lipid turnover in *Cunninghamella echinulata*. *Enzyme and microbial technology, 40*(5), 1321-1327.

Fakas, S., Papanikolaou, S., Batsos, A., Galiotou-Panayotou, M., Mallouchos, A., & Aggelis, G. (2009). Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina*. *Biomass and Bioenergy, 33*(4), 573-580.

Faridnia, F., Burritt, D. J., Bremer, P. J., & Oey, I. (2015). Innovative approach to determine the effect of pulsed electric fields on the microstructure of whole potato tubers: Use of cell viability, microscopic images and ionic leakage measurements. *Food Research International, 77*, 556-564.

Federation, W. E., & Association, A. P. H. (2005). Standard methods for the examination of water and wastewater. *American Public Health Association (APHA): Washington, DC, USA*.

Fei, Q., Chang, H. N., Shang, L., Kim, N., & Kang, J. (2011). The effect of volatile fatty acids as a sole carbon source on lipid accumulation by *Cryptococcus albidus* for biodiesel production. *Bioresource technology, 102*(3), 2695-2701.

Feng, H., Yang, W., & Hielscher, T. (2008). Power ultrasound. *Revista de Agaroquimica y Tecnologia de Alimentos, 14*(5), 433-436.

Flisar, K., Meglic, S. H., Morelj, J., Golob, J., & Miklavcic, D. (2014). Testing a prototype pulse generator for a continuous flow system and its use for *E. coli* inactivation and microalgae lipid extraction. *Bioelectrochemistry, 100*, 44-51.

Folch, J., Lees, M., & Sloane Stanley, G. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J biol Chem, 226*(1), 497-509.

Fontanille, P., Kumar, V., Christophe, G., Nouaille, R., & Larroche, C. (2012). Bioconversion of volatile fatty acids into lipids by the oleaginous yeast *Yarrowia lipolytica*. *Bioresource technology, 114*, 443-449.

Ganapathy, B., Yahya, A., & Ibrahim, N. (2019). Bioremediation of palm oil mill effluent (POME) using indigenous *Meyerozyma guilliermondii*. *Environmental Science and Pollution Research, 26*(11), 11113-11125.

Ganeva, V., Galutzov, B., & Teissie, J. (2014). Evidence that pulsed electric field treatment enhances the cell wall porosity of yeast cells. *Applied biochemistry and biotechnology, 172*(3), 1540-1552.

Garay, L. A., Boundy-Mills, K. L., & German, J. B. (2014). Accumulation of high-value lipids in single-cell microorganisms: a mechanistic approach and future perspectives. *Journal of agricultural and food chemistry, 62*(13), 2709-2727.

Garcia, P. A., Ge, Z., Moran, J. L., & Buie, C. R. (2016). Microfluidic screening of electric fields for electroporation. *Scientific reports, 6*, 21238.

Garode, A. (2018). Isolation, characterization and identification of extracellular enzyme producer *Bacillus licheniformis* from municipal wastewater and evaluation of their biodegradability. *Biotechnology Research and Innovation*.

Garoma, T., & Shackelford, T. (2014). Electroporation of *Chlorella vulgaris* to enhance biomethane production. *Bioresource technology, 169*, 778-783.

Gawai, K., Mogha, K., & Prajapati, J. (2017). Evaluation of *B. cereus* MTCC 25641 for the Treatment of Dairy Waste Effluent. *Water Environment Research, 89*(5), 466-471.

Gerken, H. G., Donohoe, B., & Knoshaug, E. P. (2013). Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. *Planta, 237*(1), 239-253.

Ghafari, S., Aziz, H. A., Isa, M. H., & Zinatizadeh, A. A. (2009). Application of response surface methodology (RSM) to optimize coagulation–flocculation treatment of leachate using poly-aluminum chloride (PAC) and alum. *Journal of hazardous materials, 163*(2), 650-656.

Gobi, K., & Vadivelu, V. (2013). By-products of palm oil mill effluent treatment plant–A step towards sustainability. *Renewable and Sustainable Energy Reviews, 28*, 788-803.

Goettel, M., Eing, C., Gusbeth, C., Straessner, R., & Frey, W. (2013). Pulsed electric field assisted extraction of intracellular valuables from microalgae. *Algal Research, 2*(4), 401-408.

Gómez, N., García, D., Álvarez, I., Raso, J., & Condón, S. (2005). A model describing the kinetics of inactivation of *Lactobacillus plantarum* in a buffer system of different pH and in orange and apple juice. *Journal of Food Engineering, 70*(1), 7-14.

Gong, Z., Wang, Q., Shen, H., Hu, C., Jin, G., & Zhao, Z. K. (2012). Co-fermentation of cellobiose and xylose by *Lipomyces starkeyi* for lipid production. *Bioresource technology, 117*, 20-24.

Gonzalez, M. E., & Barrett, D. M. (2010). Thermal, high pressure, and electric field processing effects on plant cell membrane integrity and relevance to fruit and vegetable quality. *Journal of Food Science, 75*(7), R121-R130.

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R. K., Gowda, C. L., & Krishnamurthy, L. (2015). Plant growth promoting *rhizobia:* challenges and opportunities. *3 Biotech, 5*(4), 355-377.

Gouda, M. K., Omar, S. H., & Aouad, L. M. (2008). Single cell oil production by *Gordonia sp.* DG using agro-industrial wastes. *World Journal of Microbiology and Biotechnology, 24*(9), 1703.

Grady, C. L., Daigger, G. T., Love, N. G., & Filipe, C. D. (2011). *Biological wastewater treatment*: CRC press.

Granot, Y., & Rubinsky, B. (2010). Mathematical models of mass transfer in tissue for molecular medicine with reversible electroporation *Porous Media: Applications in Biological Systems and Biotechnology* (pp. 45-74): CRC Press.

Grimi, N., Dubois, A., Marchal, L., Jubeau, S., Lebovka, N., & Vorobiev, E. (2014). Selective extraction from microalgae *Nannochloropsis sp.* using different methods of cell disruption. *Bioresource technology, 153*, 254-259.

Günerken, E., d'Hondt, E., Eppink, M., Garcia-Gonzalez, L., Elst, K., & Wijffels, R. (2015). Cell disruption for microalgae biorefineries. *Biotechnology advances, 33*(2), 243-260.

Gupta, N., Manikandan, N. A., & Pakshirajan, K. (2018). Real-time lipid production and dairy wastewater treatment using *Rhodococcus opacus* in a bioreactor under fed-batch, continuous and continuous cell recycling modes for potential biodiesel application. *Biofuels, 9*(2), 239-245.

Gusbeth, C., Frey, W., Schwartz, T., & Rieder, A. (2009a). Critical comparison between the pulsed electric field and thermal decontamination methods of hospital wastewater. *Acta Physica Polonica-Series A General Physics, 115*(6), 1092.

Gusbeth, C., Frey, W., Volkmann, H., Schwartz, T., & Bluhm, H. (2009b). Pulsed electric field treatment for bacteria reduction and its impact on hospital wastewater. *Chemosphere, 75*(2), 228-233.

Haas, C., & Aturaliye, D. (1999). Semi-quantitative characterization of electroporation-assisted disinfection processes for inactivation of *Giardia* and *Cryptosporidium*. *Journal of applied microbiology, 86*(6), 899-905.

Haberl, S., Jarc, M., Štrancar, A., Peterka, M., Hodžić, D., & Miklavčič, D. (2013). Comparison of alkaline lysis with electroextraction and optimization of electric pulses to extract plasmid DNA from *Escherichia coli*. *The Journal of membrane biology, 246*(11), 861-867.

Halim, R., Rupasinghe, T. W., Tull, D. L., & Webley, P. A. (2014). Modelling the kinetics of lipid extraction from wet microalgal concentrate: A novel perspective on a classical process. *Chemical Engineering Journal, 242*, 234-253.

Harak, C., Meyrath, M., Romero-Brey, I., Schenk, C., Gondeau, C., Schult, P., Schnitzler, P. (2017). Tuning a cellular lipid kinase activity adapts hepatitis C virus to replication in cell culture. *Nature microbiology, 2*(3), 16247.

Harde, S., Wang, Z., Horne, M., Zhu, J., & Pan, X. (2016). Microbial lipid production from SPORL-pretreated Douglas fir by *Mortierella isabellina*. *Fuel, 175*, 64-74.

Hofmann, G. A., & Evans, G. A. (1986). Electronic genetic-physical and biological aspects of cellular electromanipulation. *IEEE Engineering in Medicine and Biology Magazine, 5*(4), 6-25.

Hu, C., Wu, S., Wang, Q., Jin, G., Shen, H., & Zhao, Z. K. (2011). Simultaneous utilization of glucose and xylose for lipid production by *Trichosporon cutaneum*. *Biotechnology for biofuels, 4*(1), 25.

Huang, G., Chen, F., Wei, D., Zhang, X., & Chen, G. (2010). Biodiesel production by microalgal biotechnology. *Applied Energy, 87*(1), 38-46.

Huang, L., Zhang, B., Gao, B., & Sun, G. (2011). Application of fishmeal wastewater as a potential low-cost medium for lipid production by *Lipomyces starkeyi* HL. *Environmental Technology, 32*(16), 1975-1981.

Hülsheger, H., Potel, J., & Niemann, E.-G. (1981). Killing of bacteria with electric pulses of high field strength. *Radiation and environmental biophysics, 20*(1), 53-65.

Hülsheger, H., Potel, J., & Niemann, E.-G. (1983). Electric field effects on bacteria and yeast cells. *Radiation and environmental biophysics, 22*(2), 149-162.

Huo, Z.-Y., Li, G.-Q., Yu, T., Feng, C., Lu, Y., Wu, Y.-H., . . . Hu, H.-Y. (2018). Cell Transport Prompts the Performance of Low-Voltage Electroporation for Cell Inactivation. *Scientific reports, 8*(1), 15832.

Hwang, J.-H., Choi, J.-A., Abou-Shanab, R. A., Min, B., Song, H., Kim, Y., Jeon, B.-H. (2011). Feasibility of hydrogen production from ripened fruits by a combined two-stage (dark/dark) fermentation system. *Bioresource technology, 102*(2), 1051-1058.

Iqbal, J., & Theegala, C. (2013). Microwave assisted lipid extraction from microalgae using biodiesel as co-solvent. *Algal Research, 2*(1), 34-42.

Islam, M. A., Ethiraj, B., Cheng, C. K., Yousuf, A., & Khan, M. M. R. (2017a). Electrogenic and anti-methanogenic properties of *Bacillus cereus* for enhanced power generation in anaerobic sludge driven microbial fuel cell. *Energy & Fuels*.

Islam, M. A., Ethiraj, B., Cheng, C. K., Yousuf, A., Thiruvenkadam, S., Prasad, R., & Rahman Khan, M. M. (2018a). Enhanced current generation using mutualistic interaction of yeast-bacterial coculture in dual chamber microbial fuel cell. *Industrial & engineering chemistry research, 57*(3), 813-821.

Islam, M. A., Karim, A., Mishra, P., Mohammad, C. K., Faizal, M., Khan, M. R., & Yousuf, A. (2019). Role of Biocatalyst in Microbial Fuel Cell Performance. *Waste to Sustainable Energy: MFCs–Prospects through Prognosis*.

Islam, M. A., Karim, A., Woon, C. W., Ethiraj, B., Cheng, C. K., Yousuf, A., & Khan, M. M. R. (2017b). Augmentation of air cathode microbial fuel cell performance using wild type *Klebsiella variicola*. *RSC advances*, *7*(8), 4798-4805.

Islam, M. A., Ong, H. R., Ethiraj, B., Cheng, C. K., & Khan, M. M. R. (2018b). Optimization of co-culture inoculated microbial fuel cell performance using response surface methodology. *Journal of environmental management*, *225*, 242-251.

Islam, M. A., Woon, C. W., Ethiraj, B., Cheng, C. K., Yousuf, A., & Khan, M. M. R. (2016). Ultrasound driven biofilm removal for stable power generation in microbial fuel cell. *Energy & Fuels*, *31*(1), 968-976.

Islam, M. A., Yousuf, A., Karim, A., Pirozzi, D., Khan, M. R., & Ab Wahid, Z. (2018c). Bioremediation of palm oil mill effluent and lipid production by *Lipomyces starkeyi:* A combined approach. *Journal of cleaner production*, *172*, 1779-1787.

Ismail, S., Idris, I., Ng, Y., & Ahmad, A. (2013). Coagulation of Palm Oil Mill Effluent (POME) at High Temperature.

Iwuagwu, J. O., & Ugwuanyi, J. O. (2014). Treatment and valorization of palm oil mill effluent through production of food grade yeast biomass. *Journal of Waste Management*, *2014*.

Jeong, D.-Y., Cho, S.-K., Shin, H.-S., & Jung, K.-W. (2015). Application of an electric field for pretreatment of a feedstock (*Laminaria japonica*) for dark fermentative hydrogen production. *Biomass and Bioenergy*, *72*, 184-188.

Jia, Q., Wei, L., Han, H., & Shen, J. (2014). Factors that influence the performance of two-chamber microbial fuel cell. *international journal of hydrogen energy*, *39*(25), 13687-13693.

Jin, G., Yang, F., Hu, C., Shen, H., & Zhao, Z. K. (2012). Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast *Rhodosporidium toruloides*. *Bioresource technology*, *111*, 378-382.

Jiru, T. M., Groenewald, M., Pohl, C., Steyn, L., Kiggundu, N., & Abate, D. (2017). Optimization of cultivation conditions for biotechnological production of lipid by *Rhodotorula kratochvilovae* (syn, *Rhodosporidium kratochvilovae*) SY89 for biodiesel preparation. *3 Biotech*, *7*(2), 145.

Jones, E. M., Cochrane, C. A., & Percival, S. L. (2015). The effect of pH on the extracellular matrix and biofilms. *Advances in wound care*, *4*(7), 431-439.

Jordan, C. A., Neumann, E., & Sowers, A. E. (2013). *Electroporation and electrofusion in cell biology*: Springer Science & Business Media.

Joseph, V. R., & Melkote, S. N. (2009). Statistical adjustments to engineering models. *Journal of Quality Technology*, *41*(4), 362.

Joseph, V. R., & Yan, H. (2015). Engineering-driven statistical adjustment and calibration. *Technometrics*, *57*(2), 257-267.

Joshi, R., & Schoenbach, K. (2000). Electroporation dynamics in biological cells subjected to ultrafast electrical pulses: a numerical simulation study. *Physical review E, 62*(1), 1025.

Kakkad, H., Khot, M., Zinjarde, S., & RaviKumar, A. (2015). Biodiesel production by direct in situ transesterification of an oleaginous tropical mangrove fungus grown on untreated agro-residues and evaluation of its fuel properties. *BioEnergy Research, 8*(4), 1788-1799.

Kandušer, M., Belič, A., Čorović, S., & Škrjanc, I. (2017). Modular Serial Flow Through device for pulsed electric field treatment of the liquid samples. *Scientific reports, 7*(1), 8115.

Kandušer, M., & Miklavčič, D. (2009). Electroporation in biological cell and tissue: an overview *Electrotechnologies for extraction from food plants and biomaterials* (pp. 1-37): Springer.

Karatay, S. E., & Dönmez, G. (2010). Improving the lipid accumulation properties of the yeast cells for biodiesel production using molasses. *Bioresource technology, 101*(20), 7988-7990.

Karim, A., Islam, M. A., Mohammad Faizal, C. K., Yousuf, A., Howarth, M., Dubey, B. N., . . . Khan, M. M. R. (2018a). Enhanced biohydrogen-production from citrus wastewater using anaerobic sludge pretreated by electroporation technique. *Industrial & engineering chemistry research*.

Karim, A., Islam, M. A., Mohammad Faizal, C. K., Yousuf, A., Howarth, M., Dubey, B. N., . . . Rahman Khan, M. M. (2018b). Enhanced biohydrogen production from citrus wastewater using anaerobic sludge pretreated by an electroporation technique. *Industrial & engineering chemistry research, 58*(2), 573-580.

Karim, A., Islam, M. A., Yousuf, A., Khan, M. M. R., & Faizal, C. K. M. (2019). Microbial Lipid Accumulation through Bioremediation of Palm Oil Mill Wastewater by *Bacillus cereus*. *ACS Sustainable Chemistry & Engineering, 7*(17), 14500-14508.

Karim, A., Yousuf, A., Islam, M. A., Naif, Y. H., Faizal, C. K. M., Alam, M. Z., & Pirozzi, D. (2018c). Microbial lipid extraction from Lipomyces starkeyi using irreversible electroporation. *Biotechnology Progress*.

Karim, A., Yousuf, A., Islam, M. A., Naif, Y. H., Faizal, C. K. M., Alam, M. Z., & Pirozzi, D. (2018d). Microbial lipid extraction from *Lipomyces starkeyi* using irreversible electroporation. *Biotechnology progress, 34*(4), 838-845.

Khongkhaem, P., Suttinun, O., Intasiri, A., Pinyakong, O., & Luepromchai, E. (2016). Degradation of Phenolic Compounds in Palm Oil Mill Effluent by Silica-Immobilized Bacteria in Internal Loop Airlift Bioreactors. *CLEAN–Soil, Air, Water*.

Khot, M., Kamat, S., Zinjarde, S., Pant, A., Chopade, B., & RaviKumar, A. (2012). Single cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel. *Microbial Cell Factories, 11*(1), 71.

Kim, C., Song, Y. E., Lee, C. R., Jeon, B.-H., & Kim, J. R. (2016). Glycerol-fed microbial fuel cell with a co-culture of *Shewanella oneidensis* MR-1 and *Klebsiella pneumonae* J2B. *Journal of industrial microbiology & biotechnology, 43*(10), 1397-1403.

Kim, Y.-H., Park, S., Kim, M. H., Choi, Y.-K., Yang, Y.-H., Kim, H. J., . . . Lee, S. H. (2013). Ultrasound-assisted extraction of lipids from *Chlorella vulgaris* using [Bmim][MeSO4]. *Biomass and Bioenergy, 56*, 99-103.

Kinosita, K., & Tsong, T. Y. (1979). Voltage-induced conductance in human erythrocyte membranes. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 554*(2), 479-497.

Kirubakaran, M., & Selvan, V. A. M. (2018). A comprehensive review of low cost biodiesel production from waste chicken fat. *Renewable and Sustainable Energy Reviews, 82*, 390-401.

Kollár, R., Reinhold, B. B., Petráková, E., Yeh, H. J., Ashwell, G., Drgonová, J., Cabib, E. (1997). Architecture of the yeast cell wall β (1→ 6)-glucan interconnects mannoprotein, β (1→ 3)-glucan, and chitin. *Journal of Biological Chemistry, 272*(28), 17762-17775.

Kosa, M., & Ragauskas, A. J. (2011). Lipids from heterotrophic microbes: advances in metabolism research. *Trends in biotechnology, 29*(2), 53-61.

Kotnik, T., Frey, W., Sack, M., Meglič, S. H., Peterka, M., & Miklavčič, D. (2015). Electroporation-based applications in biotechnology. *Trends in biotechnology, 33*(8), 480-488.

Koutinas, A. A., Vlysidis, A., Pleissner, D., Kopsahelis, N., Garcia, I. L., Kookos, I. K., . . . Lin, C. S. K. (2014). Valorization of industrial waste and by-product streams via fermentation for the production of chemicals and biopolymers. *Chemical Society Reviews, 43*(8), 2587-2627.

Kraisintu, P., Yongmanitchai, W., & Limtong, S. (2010). Selection and optimization for lipid production of a newly isolated oleaginous yeast, *Rhodosporidium toruloides* DMKU3-TK16. *Kasetsart Journal (Natural Science), 44*(1), 436-445.

Kreith, F. (1999). *Handbook of solid waste management*.

Kube, M., Spedding, B., Gao, L., Fan, L., & Roddick, F. (2020). Nutrient removal by alginate-immobilised *Chlorella vulgaris*: Response to different wastewater matrices. *Journal of Chemical Technology & Biotechnology*.

Kumar, M., Rathour, R., Gupta, J., Pandey, A., Gnansounou, E., & Thakur, I. S. (2020). Bacterial production of fatty acid and biodiesel: opportunity and challenges *Refining Biomass Residues for Sustainable Energy and Bioproducts* (pp. 21-49): Elsevier.

Kumar, M., Sundaram, S., Gnansounou, E., Larroche, C., & Thakur, I. S. (2018). Carbon dioxide capture, storage and production of biofuel and biomaterials by bacteria: A review. *Bioresource technology, 247*, 1059-1068.

Kumar, S., Gupta, N., & Pakshirajan, K. (2015). Simultaneous lipid production and dairy wastewater treatment using *Rhodococcus opacus* in a batch bioreactor for potential biodiesel application. *Journal of environmental chemical engineering, 3*(3), 1630-1636.

Kumar, S. J., Kumar, G. V., Dash, A., Scholz, P., & Banerjee, R. (2017). Sustainable green solvents and techniques for lipid extraction from microalgae: A review. *Algal Research, 21*, 138-147.

Kumar, V. L., & Singhal, A. (2009). Germinating seeds of the mung bean, *Vigna radiata* (Fabaceae), as a model for the preliminary evaluation of cytotoxic effects of drugs. *Biocell, 33*(1), 19-24.

Lalaurette, E., Thammannagowda, S., Mohagheghi, A., Maness, P.-C., & Logan, B. E. (2009). Hydrogen production from cellulose in a two-stage process combining fermentation and electrohydrogenesis. *international journal of hydrogen energy, 34*(15), 6201-6210.

Lanka, S., & Pydipalli, M. (2018a). Reduction of organic load from palm oil mill effluent (POME) using selected fungal strains isolated from POME dump sites. *Afr. J. Biotechnol, 17*(36), 1138-1145.

Lanka, S., & Pydipalli, M. (2018b). Reduction of organic load from palm oil mill effluent (POME) using selected fungal strains isolated from POME dump sites. *African Journal of Biotechnology, 17*(36), 1138-1145.

Lee, A. K., Lewis, D. M., & Ashman, P. J. (2012). Disruption of microalgal cells for the extraction of lipids for biofuels: processes and specific energy requirements. *Biomass and Bioenergy, 46*, 89-101.

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., & Oh, H.-M. (2010). Comparison of several methods for effective lipid extraction from microalgae. *Bioresource technology, 101*(1), S75-S77.

Lee, S. J., Yoon, B.-D., & Oh, H.-M. (1998). Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. *Biotechnology techniques, 12*(7), 553-556.

Lee, S. Y., Cho, J. M., Chang, Y. K., & Oh, Y.-K. (2017). Cell disruption and lipid extraction for microalgal biorefineries: A review. *Bioresource technology, 244*, 1317-1328.

Lee, Z.-K., Li, S.-L., Lin, J.-S., Wang, Y.-H., Kuo, P.-C., & Cheng, S.-S. (2008). Effect of pH in fermentation of vegetable kitchen wastes on hydrogen production under a thermophilic condition. *international journal of hydrogen energy, 33*(19), 5234-5241.

Lek, B. L. C., Peter, A. P., Chong, K. H. Q., Ragu, P., Sethu, V., Selvarajoo, A., & Arumugasamy, S. K. (2018). Treatment of palm oil mill effluent (POME) using chickpea (*Cicer arietinum*) as a natural coagulant and flocculant: Evaluation, process optimization and characterization of chickpea powder. *Journal of environmental chemical engineering, 6*(5), 6243-6255.

Li, Yu, H.-Q., & He, Z. (2014). Towards sustainable wastewater treatment by using microbial fuel cells-centered technologies. *Energy & Environmental Science, 7*(3), 911-924.

Li, G., Chen, J., Yan, W., & Sang, N. (2017). A comparison of the toxicity of landfill leachate exposure at the seed soaking and germination stages on *Zea mays L.(maize)*. *Journal of Environmental Sciences, 55*, 206-213.

Liang, Y., Sarkany, N., & Cui, Y. (2009). Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology letters, 31*(7), 1043-1049.

Liau, B.-C., Shen, C.-T., Liang, F.-P., Hong, S.-E., Hsu, S.-L., Jong, T.-T., & Chang, C.-M. J. (2010). Supercritical fluids extraction and anti-solvent purification of carotenoids from microalgae and associated bioactivity. *The Journal of Supercritical Fluids, 55*(1), 169-175.

Liddell, J. (1994). Solvent extraction processes for biological products *Engineering Processes for Bioseparations*: Butterworth-Heinemann Ltd, Oxford.

Liew, W. L., Kassim, M. A., Muda, K., Loh, S. K., & Affam, A. C. (2015). Conventional methods and emerging wastewater polishing technologies for palm oil mill effluent treatment: a review. *Journal of environmental management, 149*, 222-235.

Lilly, V. G., & Barnett, H. L. (1951). Physiology of the fungi. *Physiology of the fungi.*

Lindmark, J., Lagerkvist, A., Nilsson, E., Thorin, E., & Dahlquist, E. (2014). Evaluating the effects of electroporation pre-treatment on the biogas yield from ley crop silage. *Applied biochemistry and biotechnology, 174*(7), 2616-2625.

Liu, D., Ding, L., Sun, J., Boussetta, N., & Vorobiev, E. (2016). Yeast cell disruption strategies for recovery of intracellular bio-active compounds—A review. *Innovative Food Science & Emerging Technologies, 36*, 181-192.

Liu, G., Lin, Q., Jin, X., Wang, X., & Zhao, Y. (2010). Screening and fermentation optimization of microbial lipid-producing molds from forest soils. *African Journal of Microbiology Research, 4*(14), 1462-1468.

Loginova, K., Vorobiev, E., Bals, O., & Lebovka, N. (2011). Pilot study of countercurrent cold and mild heat extraction of sugar from sugar beets, assisted by pulsed electric fields. *Journal of Food Engineering, 102*(4), 340-347.

Loretta, O., Stephen, E., Ezeata, A., & Usman, E. (2016). In vitro Biodegradation of palm oil mill effluent (POME) by *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Aspergillus niger*. *J Bioremediat Biodegrad, 7*(361), 2.

Louhasakul, Y., Cheirsilp, B., & Prasertsan, P. (2016). Valorization of palm oil mill effluent into lipid and cell-bound lipase by marine yeast *Yarrowia lipolytica* and their application in biodiesel production. *Waste and biomass valorization, 7*(3), 417-426.

Luengo, E., Martínez, J. M., Bordetas, A., Álvarez, I., & Raso, J. (2015). Influence of the treatment medium temperature on lutein extraction assisted by pulsed electric fields from *Chlorella vulgaris*. *Innovative Food Science & Emerging Technologies, 29*, 15-22.

Luo, S., Sun, H., Ping, Q., Jin, R., & He, Z. (2016). A review of modeling bioelectrochemical systems: engineering and statistical aspects. *Energies, 9*(2), 111.

Madani, M., Enshaeieh, M., & Abdoli, A. (2017). Single cell oil and its application for biodiesel production. *Process Safety and Environmental Protection, 111*, 747-756.

Madani, S., Gheshlaghi, R., Mahdavi, M. A., Sobhani, M., & Elkamel, A. (2015). Optimization of the performance of a double-chamber microbial fuel cell through factorial design of experiments and response surface methodology. *Fuel, 150*, 434-440.

Magdouli, S., Brar, S. K., & Blais, J.-F. (2016). Co-culture for lipid production: advances and challenges. *Biomass and Bioenergy, 92*, 20-30.

Mahmood, W. M. A. W., Theodoropoulos, C., & Gonzalez-Miquel, M. (2017). Enhanced microalgal lipid extraction using bio-based solvents for sustainable biofuel production. *Green Chemistry, 19*(23), 5723-5733.

Manzoor, F., Karbassi, A., & Golzary, A. (2019). Removal of Heavy Metal Contaminants from Wastewater by Using *Chlorella vulgaris* Beijerinck: A Review. *Current Environmental Management (Formerly: Current Environmental Engineering), 6*(3), 174-187.

Marina, B., Noor, A. I., & Aweng, E. R. (2013). Biodegradation of oily wastewater by pure culture of *Bacillus cereus*. *Journal of Agricultural and Biological Science, 8*(2), 108-115.

Marjakangas, J. M., Lakaniemi, A.-M., Koskinen, P. E., Chang, J.-S., & Puhakka, J. A. (2015). Lipid production by eukaryotic microorganisms isolated from palm oil mill effluent. *Biochemical Engineering Journal, 99*, 48-54.

Marquez, V., Mittal, G., & Griffiths, M. (1997). Destruction and inhibition of bacterial spores by high voltage pulsed electric field. *Journal of Food Science, 62*(2), 399-401.

Martin, O., Qin, B., Chang, F., Barbosa-Cánovas, G., & Swanson, B. (1997). Inactivation of *Escherichia coli* in skim milk by high intensity pulsed electric fields. *Journal of Food Process Engineering, 20*(4), 317-336.

Masi, M., Refregiers, M., Pos, K. M., & Pages, J.-M. (2017). Mechanisms of envelope permeability and antibiotic influx and efflux in Gram-negative bacteria. *Nature microbiology, 2*(3), 17001.

Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: a review. *Renewable and Sustainable Energy Reviews, 14*(1), 217-232.

Mehta, P., Singh, D., Saxena, R., Rani, R., Gupta, R. P., Puri, S. K., & Mathur, A. S. (2018). High-value coproducts from algae—An innovational way to deal with advance algal industry *Waste to wealth* (pp. 343-363): Springer.

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., & Xian, M. (2009). Biodiesel production from oleaginous microorganisms. *Renewable Energy, 34*(1), 1-5.

Meullemiestre, A., Breil, C., Abert-Vian, M., & Chemat, F. (2016). Microwave, ultrasound, thermal treatments, and bead milling as intensification techniques for extraction of lipids from oleaginous *Yarrowia lipolytica* yeast for a biojetfuel application. *Bioresource technology, 211*, 190-199.

Meullemiestre, A., Breil, C., Abert-Vian, M., & Chemat, F. (2017a). Manothermosonication as a useful tool for lipid extraction from oleaginous microorganisms. *Ultrasonics sonochemistry, 37*, 216-221.

Meullemiestre, A., Breil, C., Abert-Vian, M., & Chemat, F. (2017b). Manothermosonication as a useful tool for lipid extraction from oleaginous microorganisms. *Ultrasonics sonochemistry*.

Middelberg, A. P. (1995). Process-scale disruption of microorganisms. *Biotechnology advances, 13*(3), 491-551.

Miklavčič, D. (2012). Network for development of electroporation-based technologies and treatments: COST TD1104. *The Journal of membrane biology, 245*(10), 591-598.

Miklavčič, D., Šemrov, D., Mekid, H., & Mir, L. M. (2000). A validated model of in vivo electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1523*(1), 73-83.

Milano, J., Ong, H. C., Masjuki, H., Chong, W., Lam, M. K., Loh, P. K., & Vellayan, V. (2016). Microalgae biofuels as an alternative to fossil fuel for power generation. *Renewable and Sustainable Energy Reviews, 58*, 180-197.

Minhas, A. K., Hodgson, P., Barrow, C. J., & Adholeya, A. (2016). A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids. *Frontiers in microbiology, 7*, 546.

Mir, L. M., & Orlowski, S. (1999). Mechanisms of electrochemotherapy. *Advanced drug delivery reviews, 35*(1), 107-118.

Mishra, P., Ameen, F., Zaid, R. M., Singh, L., Ab Wahid, Z., Islam, M. A., . . . Al Nadhari, S. (2019). Relative effectiveness of substrate-inoculum ratio and initial pH on hydrogen production from palm oil mill effluent: Kinetics and statistical optimization. *Journal of cleaner production, 228*, 276-283.

Mlaik, N., Bouzid, J., Hassan, I. B., Woodward, S., Elbahri, L., & Mechichi, T. (2015). Unhairing wastewater treatment by *Bacillus pumilus* and *Bacillus cereus*. *Desalination and Water Treatment, 54*(3), 683-689.

Moen, G., Stensel, H. D., Lepistö, R., & Ferguson, J. F. (2003). Effect of solids retention time on the performance of thermophilic and mesophilic digestion of combined municipal wastewater sludges. *Water Environment Research, 75*(6), 539-548.

Mohammed, R. R., Ketabchi, M. R., & McKay, G. (2014). Combined magnetic field and adsorption process for treatment of biologically treated palm oil mill effluent (POME). *Chemical Engineering Journal, 243*, 31-42.

Mosqueda-Melgar, J., Raybaudi-Massilia, R. M., & Martín-Belloso, O. (2007). Influence of treatment time and pulse frequency on *Salmonella Enteritidis*, *Escherichia coli* and *Listeria monocytogenes* populations inoculated in melon and watermelon juices treated by pulsed electric fields. *International Journal of Food Microbiology, 117*(2), 192-200.

Mujtaba, G., Rizwan, M., Kim, G., & Lee, K. (2018). Removal of nutrients and COD through co-culturing activated sludge and immobilized *Chlorella vulgaris*. *Chemical Engineering Journal, 343*, 155-162.

Muniraj, I. K., Xiao, L., Hu, Z., Zhan, X., & Shi, J. (2013). Microbial lipid production from potato processing wastewater using oleaginous filamentous fungi *Aspergillus oryzae*. *Water research, 47*(10), 3477-3483.

Nanganuru, H. Y., Korrapati, N., & Prakash, B. M. (2012). Studies on the potential of *Bacillus subtilis* in the biodegradation of engine oil. *Journal of Chemical, Biological and Physical Sciences (JCBPS), 2*(3), 1599.

Narsetti, R., Curry, R. D., McDonald, K. F., Clevenger, T. E., & Nichols, L. M. (2006). Microbial inactivation in water using pulsed electric fields and magnetic pulse compressor technology. *IEEE Transactions on Plasma Science, 34*(4), 1386-1393.

Nayak, M., Karemore, A., & Sen, R. (2016). Performance evaluation of microalgae for concomitant wastewater bioremediation, CO_2 biofixation and lipid biosynthesis for biodiesel application. *Algal Research, 16*, 216-223.

Ng, Y. S., Lim, C. R., & Chan, D. J. C. (2016). Development of treated palm oil mill effluent (POME) culture medium for plant tissue culture of *Hemianthus callitrichoides*. *Journal of environmental chemical engineering, 4*(4, Part B), 4890-4896. doi:http://dx.doi.org/10.1016/j.jece.2016.05.004

Nizami, A., Rehan, M., Waqas, M., Naqvi, M., Ouda, O., Shahzad, K., Ismail, I. (2017). Waste biorefineries: Enabling circular economies in developing countries. *Bioresource technology, 241*, 1101-1117.

Nur, M. (2012). Potential of palm oil mill effluent (POME) as medium growth of *Chlorella sp* for bioenergy production. *Potential of Palm Oil Mill Effluent (POME) as Medium Growth of Chlorella sp for Bioenergy Production, 3*(2), 67-74.

Nur, M. (2014). Lipid extraction of microalga *Chlorella sp.* cultivated in palm oil mill effluent (POME) medium. *World Applied Sciences Journal, 31*(5), 959-967.

Nur, M. A., & Hadiyanto, H. (2015). Enhancement of *Chlorella vulgaris* biomass cultivated in POME medium as biofuel feedstock under mixotrophic conditions. *Journal of Engineering and Technological Sciences, 47*(5), 487-497.

Nwoko, O. C., Ogunyemi, S., & Nkwocha, E. (2010). Effect of pre-treatment of Palm oil Mill effluent (POME) and Cassava Mill Effluent (CME) on the Growth of Tomato (*Lycopersicum esculentum*). *Journal of Applied Sciences and Environmental Management, 14*(1).

Nwuche, C. O., Aoyagi, H., & Ogbonna, J. C. (2014a). Treatment of palm oil mill effluent by a microbial consortium developed from compost soils. *International scholarly research notices, 2014*.

Nwuche, C. O., Ekpo, D. C., Eze, C. N., Aoyagi, H., & Ogbonna, J. C. (2014b). Use of palm oil mill effluent as medium for cultivation of *Chlorella sorokiniana*. *Biotechnology Journal International*, 305-316.

Ogunwenmo, K., Oyelana, O., Ibidunmoye, O., Anyasor, G., & Ogunnowo, A. (2010). Effects of brewery, textile and paint effluent on seed germination of leafy vegetables-*Amaranthus hybridus* and *Celosia argentea* (*Amaranthaceae*). *J Biol Sci, 10*(2), 151-156.

Ohimain, E. I., & Izah, S. C. (2017). A review of biogas production from palm oil mill effluents using different configurations of bioreactors. *Renewable and Sustainable Energy Reviews, 70*, 242-253.

Ojo, E. O., Auta, H., Baganz, F., & Lye, G. J. (2015). Design and parallelisation of a miniature photobioreactor platform for microalgal culture evaluation and optimisation. *Biochemical Engineering Journal, 103*, 93-102.

Onumaegbu, C., Mooney, J., Alaswad, A., & Olabi, A. (2018). Pre-treatment methods for production of biofuel from microalgae biomass. *Renewable and Sustainable Energy Reviews, 93*, 16-26.

Organization, W. H. (2014). *Antimicrobial resistance: 2014 global report on surveillance*: World Health Organization.

Ortiz-Martínez, V., Salar-García, M., De Los Ríos, A., Hernández-Fernández, F., Egea, J., & Lozano, L. (2015). Developments in microbial fuel cell modeling. *Chemical Engineering Journal, 271*, 50-60.

Oswal, N., Sarma, P., Zinjarde, S., & Pant, A. (2002a). Palm oil mill effluent treatment by a tropical marine yeast. *Bioresource technology, 85*(1), 35-37.

Oswal, N., Sarma, P. M., Zinjarde, S. S., & Pant, A. (2002b). Palm oil mill effluent treatment by a tropical marine yeast. *Bioresource technology, 85*(1), 35-37. doi:http://dx.doi.org/10.1016/S0960-8524(02)00063-9

Padmaperuma, G., Kapoore, R. V., Gilmour, D. J., & Vaidyanathan, S. (2018). Microbial consortia: a critical look at microalgae co-cultures for enhanced biomanufacturing. *Critical reviews in biotechnology*, *38*(5), 690-703.

Pan, L.-X., Yang, D.-F., Shao, L., Li, W., Chen, G.-G., & Liang, Z.-Q. (2009). Isolation of the oleaginous yeasts from the soil and studies of their lipid-producing capacities. *Food technology and Biotechnology*, *47*(2), 215-220.

Pandey, A., Singh, M. P., Kumar, S., & Srivastava, S. (2019). Phycoremediation of Persistent Organic Pollutants from Wastewater: Retrospect and Prospects *Application of Microalgae in Wastewater Treatment* (pp. 207-235): Springer.

Pandey, S., Nautiyal, B., & Sharma, C. (2008). Pollution level in distillery effluent and its phytotoxic effect on seed germination and early growth of maize and rice. *Journal of Environmental Biology*, *29*(2), 267.

Papazi, A., Andronis, E., Ioannidis, N. E., Chaniotakis, N., & Kotzabasis, K. (2012). High yields of hydrogen production induced by meta-substituted dichlorophenols biodegradation from the green alga *Scenedesmus obliquus*. *PLoS One*, *7*(11), e49037.

Park, J.-Y., Park, M. S., Lee, Y.-C., & Yang, J.-W. (2015). Advances in direct transesterification of algal oils from wet biomass. *Bioresource technology*, *184*, 267-275.

Patel, A., Karageorgou, D., Rova, E., Katapodis, P., Rova, U., Christakopoulos, P., & Matsakas, L. (2020). An Overview of Potential Oleaginous Microorganisms and Their Role in Biodiesel and Omega-3 Fatty Acid-Based Industries. *Microorganisms*, *8*(3), 434.

Patel, A., Mikes, F., & Matsakas, L. (2018). An overview of current pretreatment methods used to improve lipid extraction from oleaginous microorganisms. *Molecules*, *23*(7), 1562.

Pillet, F., Formosa-Dague, C., Baaziz, H., Dague, E., & Rols, M.-P. (2016). Cell wall as a target for bacteria inactivation by pulsed electric fields. *Scientific reports*, *6*, 19778.

Pinto, R., Tartakovsky, B., & Srinivasan, B. (2012). Optimizing energy productivity of microbial electrochemical cells. *Journal of Process Control*, *22*(6), 1079-1086.

Pinzi, S., Leiva, D., López-García, I., Redel-Macías, M. D., & Dorado, M. P. (2014). Latest trends in feedstocks for biodiesel production. *Biofuels, Bioproducts and Biorefining*, *8*(1), 126-143.

Pirozzi, D., Ausiello, A., Zuccaro, G., Sannino, F., & Yousuf, A. (2013). *Culture of oleaginous yeasts in dairy industry wastewaters to obtain lipids suitable for the production of II-generation Biodiesel.* Paper presented at the Proceedings of World Academy of Science, Engineering and Technology.

Pirozzi, D., Ausielloa, A., Yousuf, A., Zuccaroa, G., & Toscanoa, G. (2014). Exploitation of Oleaginous Yeasts for the Production of Microbial Oils from Agricultural Biomass. *CHEMICAL ENGINEERING, 37*.

Pirozzi, D., Fiorentino, N., Impagliazzo, A., Sannino, F., Yousuf, A., Zuccaro, G., & Fagnano, M. (2015). Lipid production from *Arundo donax* grown under different agronomical conditions. *Renewable Energy, 77*, 456-462.

Pleissner, D., Lau, K. Y., & Lin, C. S. K. (2017). Utilization of food waste in continuous flow cultures of the heterotrophic microalga *Chlorella pyrenoidosa* for saturated and unsaturated fatty acids production. *Journal of cleaner production, 142*, 1417-1424.

Pliquett, U., Joshi, R., Sridhara, V., & Schoenbach, K. (2007). High electrical field effects on cell membranes. *Bioelectrochemistry, 70*(2), 275-282.

Powers, M. J., Sanabria-Valentín, E., Bowers, A. A., & Shank, E. A. (2015). Inhibition of cell differentiation in *Bacillus subtilis* by *Pseudomonas protegens*. *Journal of bacteriology, 197*(13), 2129-2138.

Qadeer, S., Mahmood, S., Anjum, M., Ilyas, N., Ali, Z., & Khalid, A. (2018). Synchronization of lipid-based biofuel production with waste treatment using oleaginous bacteria: A biorefinery concept. *Process Safety and Environmental Protection, 115*, 99-107.

Qi, F., Pei, H., Ma, G., Zhang, S., & Mu, R. (2016). Improving productivity and quality of biodiesel from *Chlorella vulgaris* SDEC-3M through customized process designs. *Energy conversion and management, 129*, 100-107.

Qin, B.-L., Zhang, Q., Barbosa-Canovas, G. V., Swanson, B. G., & Pedrow, P. D. (1994). Inactivation of microorganisms by pulsed electric fields of different voltage waveforms. *IEEE Transactions on Dielectrics and Electrical Insulation, 1*(6), 1047-1057.

Rajendhran, J., Krishnakumar, V., & Gunasekaran, P. (2002). Optimization of a fermentation medium for the production of *Penicillin* G acylase from *Bacillus sp*. *Letters in applied microbiology, 35*(6), 523-527.

Rajesh, P., Jadhav, D., & Ghangrekar, M. (2015). Improving performance of microbial fuel cell while controlling methanogenesis by *Chaetoceros* pretreatment of anodic inoculum. *Bioresource technology, 180*, 66-71.

Ramos, C., & Teissié, J. (2000). Electrofusion: a biophysical modification of cell membrane and a mechanism in exocytosis. *Biochimie, 82*(5), 511-518.

Rao, A. R., Dayananda, C., Sarada, R., Shamala, T., & Ravishankar, G. (2007). Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents. *Bioresource technology, 98*(3), 560-564.

Raschke, D. (2010). *Pulsed electric fields-Influence on physiology, structure and extraction processes of the oleaginous yeast Waltomyces lipofer*. Dissertation. Universitat Berlin. Berlin. Germany.

Read, S. T., Dutta, P., Bond, P. L., Keller, J., & Rabaey, K. (2010). Initial development and structure of biofilms on microbial fuel cell anodes. *BMC microbiology, 10*(1), 1. doi:10.1186/1471-2180-10-98

Ren, X., Zhao, X., Turcotte, F., Deschênes, J.-S., Tremblay, R., & Jolicoeur, M. (2017). Current lipid extraction methods are significantly enhanced adding a water treatment step in *Chlorella protothecoides*. *Microbial Cell Factories, 16*(1), 26.

Reno, U., Regaldo, L., & Gagneten, A. M. (2020). Circular Economy and Agro-Industrial Wastewater: Potential of Microalgae in Bioremediation Processes *Valorisation of Agro-industrial Residues–Volume I: Biological Approaches* (pp. 111-129): Springer.

Rieder, A., Schwartz, T., Schön-Hölz, K., Marten, S. M., Süß, J., Gusbeth, C., . . . Frey, W. (2008). Molecular monitoring of inactivation efficiencies of bacteria during pulsed electric field treatment of clinical wastewater. *Journal of applied microbiology, 105*(6), 2035-2045.

Rittmann, B. E., Lee, H.-s., Zhang, H., Alder, J., Banaszak, J. E., & Lopez, R. (2008). Full-scale application of focused-pulsed pre-treatment for improving biosolids digestion and conversion to methane. *Water Science and Technology, 58*(10), 1895-1901.

Rodolfi, L., Biondi, N., Guccione, A., Bassi, N., D'Ottavio, M., Arganaraz, G., & Tredici, M. R. (2017). Oil and eicosapentaenoic acid production by the diatom *Phaeodactylum tricornutum* cultivated outdoors in Green Wall Panel (GWP®) reactors. *Biotechnology and bioengineering, 114*(10), 2204-2210.

Rout, P. R., Dash, R. R., Bhunia, P., & Rao, S. (2018). Role of *Bacillus cereus* GS-5 strain on simultaneous nitrogen and phosphorous removal from domestic wastewater in an inventive single unit multi-layer packed bed bioreactor. *Bioresource technology, 262*, 251-260.

Ruan, Z., Zanotti, M., Archer, S., Liao, W., & Liu, Y. (2014). Oleaginous fungal lipid fermentation on combined acid-and alkali-pretreated corn stover hydrolysate for advanced biofuel production. *Bioresource technology, 163*, 12-17.

Rude, M. A., & Schirmer, A. (2009). New microbial fuels: a biotech perspective. *Current opinion in microbiology, 12*(3), 274-281.

Ryckebosch, E., Muylaert, K., & Foubert, I. (2012). Optimization of an analytical procedure for extraction of lipids from microalgae. *Journal of the American Oil Chemists' Society, 89*(2), 189-198.

Sabirova, J. S., Haddouche, R., Van Bogaert, I., Mulaa, F., Verstraete, W., Timmis, K., Soetaert, W. (2011). The 'LipoYeasts' project: using the oleaginous yeast *Yarrowia lipolytica* in combination with specific bacterial genes for the bioconversion of lipids, fats and oils into high-value products. *Microbial biotechnology, 4*(1), 47-54.

Sack, M., Sigler, J., Frenzel, S., Eing, C., Arnold, J., Michelberger, T., . . . Müller, G. (2010). Research on industrial-scale electroporation devices fostering the extraction of substances from biological tissue. *Food Engineering Reviews, 2*(2), 147-156.

Sadabad, H. R., & Gholikandi, G. B. (2017). Harvesting direct electricity from municipal waste-activated sludge simultaneous with its aerobic stabilization process: Investigation and optimization. *Journal of environmental chemical engineering, 5*(1), 1174-1185.

Saenge, C., Cheirsilp, B., Suksaroge, T. T., & Bourtoom, T. (2011). Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. *Process Biochemistry, 46*(1), 210-218.

Safavi, S. M., & Unnthorsson, R. (2017). Enhanced methane production from pig slurry with pulsed electric field pre-treatment. *Environmental Technology*, 1-11.

Sahena, F., Zaidul, I., Jinap, S., Karim, A., Abbas, K., Norulaini, N., & Omar, A. (2009). Application of supercritical CO_2 in lipid extraction–A review. *Journal of Food Engineering, 95*(2), 240-253.

Saldaña, G., Álvarez, I., Condón, S., & Raso, J. (2014). Microbiological aspects related to the feasibility of PEF technology for food pasteurization. *Critical reviews in food science and nutrition, 54*(11), 1415-1426.

Sale, A., & Hamilton, W. (1967). Effects of high electric fields on microorganisms: I. Killing of bacteria and yeasts. *Biochimica et Biophysica Acta (BBA)-General Subjects, 148*(3), 781-788.

Salerno, M. B., Lee, H.-S., Parameswaran, P., & Rittmann, B. E. (2009). Using a pulsed electric field as a pretreatment for improved biosolids digestion and methanogenesis. *Water Environment Research, 81*(8), 831-839.

Salgado, J. M., Abrunhosa, L., Venâncio, A., Domínguez, J. M., & Belo, I. (2016). Combined bioremediation and enzyme production by *Aspergillus sp.* in olive mill and winery wastewaters. *International Biodeterioration & Biodegradation, 110*, 16-23. doi:http://dx.doi.org/10.1016/j.ibiod.2015.12.011

Samorì, G., Samorì, C., Guerrini, F., & Pistocchi, R. (2013). Growth and nitrogen removal capacity of *Desmodesmus communis* and of a natural microalgae consortium in a batch culture system in view of urban wastewater treatment: part I. *Water research, 47*(2), 791-801.

Sangeetha, J., Thangadurai, D., David, M., & Abdullah, M. A. (2016). Integrated Algal Industrial Waste Treatment and Bioenergy Co-Generation *Environmental Biotechnology* (pp. 175-246): Apple Academic Press.

Sapci, Z., & Morken, J. (2014). The effect of algae species on biodiesel and biogas production observed by using a data model combines algae cultivation with an anaerobic digestion (ACAD) and a biodiesel process. *Energy conversion and management, 79*, 519-524.

Sathishkumar, M., Binupriya, A. R., Baik, S. H., & Yun, S. E. (2008). Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium isolated from hydrocarbon contaminated areas. *CLEAN–Soil, Air, Water, 36*(1), 92-96.

Sati, H., Mitra, M., Mishra, S., & Baredar, P. (2019). Microalgal lipid extraction strategies for biodiesel production: A review. *Algal Research, 38*, 101413.

Saulis, G. (2010). Electroporation of cell membranes: the fundamental effects of pulsed electric fields in food processing. *Food Engineering Reviews, 2*(2), 52-73.

Saunders, J. A., Matthews, B. F., & Miller, P. D. (1989). Plant gene transfer using electrofusion and electroporation. *Electroporation and Electrofusion in Cell Biology*, 343-354.

Schneider, T., Graeff-Hönninger, S., French, W., Hernandez, R., Merkt, N., Claupein, W., . . . Pham, P. (2013). Lipid and carotenoid production by oleaginous red yeast *Rhodotorula glutinis* cultivated on brewery effluents. *Energy, 61*, 34-43.

Schwenzfeier, A., Helbig, A., Wierenga, P. A., & Gruppen, H. (2013). Emulsion properties of algae soluble protein isolate from *Tetraselmis sp*. *Food Hydrocolloids, 30*(1), 258-263.

Scott, S. D., Armenta, R. E., Berryman, K. T., & Norman, A. W. (2011). Use of raw glycerol to produce oil rich in polyunsaturated fatty acids by a *thraustochytrid*. *Enzyme and microbial technology, 48*(3), 267-272.

Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Chowdhury, M. K., Parsaeimehr, A., . . . Daroch, M. (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. *Journal of Applied Phycology, 30*(1), 197-213.

Shah, S., Ahmad, A., Othman, M., & Abdullah, M. (2016). Effects of palm oil mill effluent media on cell growth and lipid content of *Nannochloropsis oculata* and *Tetraselmis suecica*. *International Journal of Green Energy, 13*(2), 200-207.

Sheng, J., Vannela, R., & Rittmann, B. (2012). Disruption of *Synechocystis* PCC 6803 for lipid extraction. *Water Science and Technology, 65*(3), 567-573.

Sheng, J., Vannela, R., & Rittmann, B. E. (2011). Evaluation of cell-disruption effects of pulsed-electric-field treatment of *Synechocystis* PCC 6803. *Environmental science & technology, 45*(8), 3795-3802.

Shoaib, A., Bhran, A., Rasmey, A.-H., & Mikky, Y. (2018). Optimization of cultural conditions for lipid accumulation by *Aspergillus wentii* Ras101 and its transesterification to biodiesel: application of response surface methodology. *3 Biotech, 8*(10), 417.

Shuba, E. S., & Kifle, D. (2018). Microalgae to biofuels:'Promising'alternative and renewable energy, review. *Renewable and Sustainable Energy Reviews, 81*, 743-755.

Singh, R., Ibrahim, M. H., Esa, N., & Iliyana, M. (2010). Composting of waste from palm oil mill: a sustainable waste management practice. *Reviews in Environmental Science and Bio/Technology*, *9*(4), 331-344.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture*, *16*(3), 144-158.

Soleimaninanadegani, M., & Manshad, S. (2014). Enhancement of Biodegradation of Palm Oil Mill Effluents by Local Isolated Microorganisms. *International scholarly research notices*, *2014*.

Spilimbergo, S., Dehghani, F., Bertucco, A., & Foster, N. R. (2003). Inactivation of bacteria and spores by pulse electric field and high pressure CO_2 at low temperature. *Biotechnology and bioengineering*, *82*(1), 118-125.

Sreeharsha, R. V., & Mohan, S. V. (2020). Obscure yet Promising Oleaginous Yeasts for Fuel and Chemical Production. *Trends in biotechnology*.

Srinophakun, P., Thanapimmetha, A., Rattanaphanyapan, K., Sahaya, T., & Saisriyoot, M. (2017). Feedstock production for third generation biofuels through cultivation of *Arthrobacter* AK19 under stress conditions. *Journal of cleaner production*, *142*, 1259-1266.

Sriwongchai, S., Pokethitiyook, P., Kruatrachue, M., Bajwa, P. K., & Lee, H. (2013). Screening of selected oleaginous yeasts for lipid production from glycerol and some factors which affect lipid production by *Yarrowia lipolytica* strains. *The Journal of Microbiology, Biotechnology and Food Sciences*, *2*(5), 2344.

Steriti, A., Rossi, R., Concas, A., & Cao, G. (2014). A novel cell disruption technique to enhance lipid extraction from microalgae. *Bioresource technology*, *164*, 70-77.

Subhash, G. V., & Mohan, S. V. (2014). Lipid accumulation for biodiesel production by oleaginous fungus *Aspergillus awamori*: Influence of critical factors. *Fuel*, *116*, 509-515.

Subhash, G. V., Rajvanshi, M., Kumar, B. N., Govindachary, S., Prasad, V., & Dasgupta, S. (2017). Carbon streaming in microalgae: extraction and analysis methods for high value compounds. *Bioresource technology*, *244*, 1304-1316.

Suganya, T., Varman, M., Masjuki, H., & Renganathan, S. (2016). Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: a biorefinery approach. *Renewable and Sustainable Energy Reviews*, *55*, 909-941.

Sugiura, K., Ishihara, M., Shimauchi, T., & Harayama, S. (1996). Physicochemical properties and biodegradability of crude oil. *Environmental science & technology*, *31*(1), 45-51.

Sun, M., Zhai, L.-F., Li, W.-W., & Yu, H.-Q. (2016). Harvest and utilization of chemical energy in wastes by microbial fuel cells. *Chemical Society Reviews*.

Tabassum, S., Zhang, Y., & Zhang, Z. (2015). An integrated method for palm oil mill effluent (POME) treatment for achieving zero liquid discharge – A pilot study. *Journal of cleaner production, 95*, 148-155. doi:http://dx.doi.org/10.1016/j.jclepro.2015.02.056

Taha, M. R., & Ibrahim, A. (2014). COD removal from anaerobically treated palm oil mill effluent (AT-POME) via aerated heterogeneous Fenton process: Optimization study. *Journal of Water Process Engineering, 1*, 8-16.

Tai, H. P., & Kim, K. P. T. (2014). Supercritical carbon dioxide extraction of Gac oil. *The Journal of Supercritical Fluids, 95*, 567-571.

Tanzi, C. D., Abert Vian, M., Ginies, C., Elmaataoui, M., & Chemat, F. (2012). Terpenes as green solvents for extraction of oil from microalgae. *Molecules, 17*(7), 8196-8205.

Tao, W., & Zhang, M. (2008). Physical methods for gene therapy, micro-and nano-manipulations: fundamentals with biomedical applications: Artech Publishing House.

Teissie, J., Eynard, N., Vernhes, M., Benichou, A., Ganeva, V., Galutzov, B., & Cabanes, P. (2002). Recent biotechnological developments of electropulsation. A prospective review. *Bioelectrochemistry, 55*(1), 107-112.

Thakur, C., Srivastava, V. C., & Mall, I. D. (2009). Electrochemical treatment of a distillery wastewater: Parametric and residue disposal study. *Chemical Engineering Journal, 148*(2), 496-505.

Thanh, L. T., Okitsu, K., Boi, L. V., & Maeda, Y. (2012). Catalytic technologies for biodiesel fuel production and utilization of glycerol: a review. *Catalysts, 2*(1), 191-222.

Thani, M., Hussin, R., Ibrahim, W., & Sulaiman, M. (1999). Industrial processes & the environment: crude palm oil industry, Handbook No. 3. *Department of Environment, Kuala Lumpur*, 7-54.

Theerachat, M., Tanapong, P., & Chulalaksananukul, W. (2017). The culture or co-culture of *Candida rugosa* and *Yarrowia lipolytica* strain rM-4A, or incubation with their crude extracellular lipase and laccase preparations, for the biodegradation of palm oil mill wastewater. *International Biodeterioration & Biodegradation, 121*, 11-18.

Thomann, R. V. (1989). Deterministic and statistical models of chemical fate in aquatic systems *Applied Mathematical Ecology* (pp. 322-351): Springer.

Tsang, Y. F., Kumar, V., Samadar, P., Yang, Y., Lee, J., Ok, Y. S., . . . Jeon, Y. J. (2019). Production of bioplastic through food waste valorization. *Environment international, 127*, 625-644.

Tsolcha, O. N., Tekerlekopoulou, A. G., Akratos, C. S., Aggelis, G., Genitsaris, S., Moustaka-Gouni, M., & Vayenas, D. V. (2017). Biotreatment of raisin and winery wastewaters and simultaneous biodiesel production using a *Leptolyngbya*-based microbial consortium. *Journal of cleaner production, 148*, 185-193.

Uldal, M., Andreas, L., & Lagerkvist, A. (2009). *Optimerad gasproduktion med elektroporation*: Svenskt gastekniskt center (SGC).

Uzel, A., Önçağ, Ö., Çoğulu, D., & Gençay, Ö. (2005). Chemical compositions and antimicrobial activities of four different Anatolian propolis samples. *Microbiological research, 160*(2), 189-195.

Valič, B., Pavlin, M., & Miklavčič, D. (2004). The effect of resting transmembrane voltage on cell electropermeabilization: a numerical analysis. *Bioelectrochemistry, 63*(1-2), 311-315.

Van Hamme, J. D., Odumeru, J. A., & Ward, O. P. (2000). Community dynamics of a mixed-bacterial culture growing on petroleum hydrocarbons in batch culture. *Canadian journal of microbiology, 46*(5), 441-450.

Vasconcelos, B., Teixeira, J. C., Dragone, G., & Teixeira, J. A. (2019). Oleaginous yeasts for sustainable lipid production—from biodiesel to surf boards, a wide range of "green" applications. *Applied microbiology and biotechnology, 103*(9), 3651-3667.

Venkataraman, A., Rosenbaum, M. A., Perkins, S. D., Werner, J. J., & Angenent, L. T. (2011). Metabolite-based mutualism between *Pseudomonas aeruginosa* PA14 and *Enterobacter aerogenes* enhances current generation in bioelectrochemical systems. *Energy & Environmental Science, 4*(11), 4550-4559.

Verma, P., & Sharma, M. (2016). Review of process parameters for biodiesel production from different feedstocks. *Renewable and Sustainable Energy Reviews, 62*, 1063-1071.

Vicente, G., Bautista, L. F., Rodríguez, R., Gutiérrez, F. J., Sádaba, I., Ruiz-Vázquez, R. M., . . . Garre, V. (2009). Biodiesel production from biomass of an oleaginous fungus. *Biochemical Engineering Journal, 48*(1), 22-27.

Vidal, O. (2014). First pulsed electric field (PEF) application at industrial scale in beet sugar industry. *Sugar Industry, 139*(64), 37-39.

Vijayaraghavan, K., Ahmad, D., & Ibrahim, M. K. B. (2006). Biohydrogen generation from jackfruit peel using anaerobic contact filter. *international journal of hydrogen energy, 31*(5), 569-579.

Wahlen, B. D., Willis, R. M., & Seefeldt, L. C. (2011). Biodiesel production by simultaneous extraction and conversion of total lipids from microalgae, cyanobacteria, and wild mixed-cultures. *Bioresource technology, 102*(3), 2724-2730.

Walde, P., Giuliani, A. M., Boicelli, C. A., & Luisi, P. L. (1990). Phospholipid-based reverse micelles. *Chemistry and physics of lipids, 53*(4), 265-288.

Wang, R., Xue, S., Zhang, D., Zhang, Q., Wen, S., Kong, D., . . . Cong, W. (2015a). Construction and characteristics of artificial consortia of *Scenedesmus obliquus*-bacteria for *S. obliquus* growth and lipid production. *Algal Research, 12*, 436-445.

Wang, Y., Yang, Y., Ma, F., Xuan, L., Xu, Y., Huo, H., . . . Dong, S. (2015b). Optimization of *Chlorella vulgaris* and bioflocculant-producing bacteria co-culture: enhancing microalgae harvesting and lipid content. *Letters in applied microbiology*, *60*(5), 497-503.

Weaver, J. C., & Chizmadzhev, Y. A. (1996). Theory of electroporation: a review. *Bioelectrochemistry and bioenergetics*, *41*(2), 135-160.

Weaver, J. C., Smith, K. C., Esser, A. T., Son, R. S., & Gowrishankar, T. (2012). A brief overview of electroporation pulse strength–duration space: A region where additional intracellular effects are expected. *Bioelectrochemistry*, *87*, 236-243.

Weiland, P. (2010). Biogas production: current state and perspectives. *Applied microbiology and biotechnology*, *85*(4), 849-860.

Wen, Q., Wu, Y., Cao, D., Zhao, L., & Sun, Q. (2009). Electricity generation and modeling of microbial fuel cell from continuous beer brewery wastewater. *Bioresource technology*, *100*(18), 4171-4175.

Wu, Y., Mittal, G., & Griffiths, M. (2005). Effect of pulsed electric field on the inactivation of microorganisms in grape juices with and without antimicrobials. *Biosystems Engineering*, *90*(1), 1-7.

Wu, Y., Zhou, S., Qin, F., Zheng, K., & Ye, X. (2010). Modeling the oxidation kinetics of Fenton's process on the degradation of humic acid. *Journal of hazardous materials*, *179*(1), 533-539.

Xu, J., Du, W., Zhao, X., Zhang, G., & Liu, D. (2013). Microbial oil production from various carbon sources and its use for biodiesel preparation. *Biofuels, Bioproducts and Biorefining*, *7*(1), 65-77.

Xu, L., Brilman, D. W. W., Withag, J. A., Brem, G., & Kersten, S. (2011). Assessment of a dry and a wet route for the production of biofuels from microalgae: energy balance analysis. *Bioresource technology*, *102*(8), 5113-5122.

Xu, P., Qiao, K., Ahn, W. S., & Stephanopoulos, G. (2016). Engineering *Yarrowia lipolytica* as a platform for synthesis of drop-in transportation fuels and oleochemicals. *Proceedings of the National Academy of Sciences*, *113*(39), 10848-10853.

Yang, X., Sun, W., Shen, H., Zhang, S., Jiao, X., & Zhao, Z. K. (2018). Expression of phosphotransacetylase in *Rhodosporidium toruloides* leading to improved cell growth and lipid production. *RSC advances*, *8*(43), 24673-24678.

Yano, K.-i., Rems, L., Kotnik, T., Miklavčič, D., Weaver, J. C., Smith, K. C., . . . Levine, Z. A. (2017). Biological Responses *Bioelectrics* (pp. 155-274): Springer.

Yeo, S. K., & Liong, M. T. (2013). Effect of electroporation on viability and bioconversion of isoflavones in mannitol-soymilk fermented by *lactobacilli* and *bifidobacteria*. *Journal of the Science of Food and Agriculture, 93*(2), 396-409.

Yousuf, A. (2012). Biodiesel from lignocellulosic biomass–Prospects and challenges. *Waste Management, 32*(11), 2061-2067.

Yousuf, A., Khan, M. R., Islam, A., Monir, M. U., Ab Wahid, Z., & Pirozzi, D. (2017a). *Application of Electroporation Technique in Biofuel Processing.* Paper presented at the MATEC Web of Conferences.

Yousuf, A., Khan, M. R., Islam, M. A., Ab Wahid, Z., & Pirozzi, D. (2017b). Technical difficulties and solutions of direct transesterification process of microbial oil for biodiesel synthesis. *Biotechnology letters, 39*(1), 13-23.

Yousuf, A., Khan , M. R., Islam, M. A., Wahid, Z. A., & Pirozzi, D. (2016). Technical difficulties and solutions of direct transesterification process of microbial oil for biodiesel synthesis. *Biotechnology letters*.

Yousuf, A., Khan, M. R., Islam, M. A., Wahid, Z. A., & Pirozzi, D. (2017c). Technical difficulties and solutions of direct transesterification process of microbial oil for biodiesel synthesis. *Biotechnology letters, 39*(1), 13-23. doi:10.1007/s10529-016-2217-x

Yousuf, A., & Pirozzi, D. (2009). *Prospect of agro-industrial residues as feedstock of biodiesel.* Paper presented at the Developments in Renewable Energy Technology (ICDRET), 2009 1st International Conference on the.

Yousuf, A., Sannino, F., Addorisio, V., & Pirozzi, D. (2010). Microbial conversion of olive oil mill wastewaters into lipids suitable for biodiesel production. *Journal of agricultural and food chemistry, 58*(15), 8630-8635.

Yousuf, A., Sultana, S., Monir, M. U., Karim, A., & Rahmaddulla, S. R. B. (2017d). Social business models for empowering the biogas technology. *Energy Sources, Part B: Economics, Planning, and Policy, 12*(2), 99-109.

Yu, Z., Zhang, X., Ren, P., Zhang, M., & Qian, J. (2012). Therapeutic potential of irreversible electroporation in sarcoma. *Expert review of anticancer therapy, 12*(2), 177-184.

Yuan, Y., Wang, S., Liu, Y., Li, B., Wang, B., & Peng, Y. (2015). Long-term effect of pH on short-chain fatty acids accumulation and microbial community in sludge fermentation systems. *Bioresource technology, 197*, 56-63.

Yusaf, T., & Al-Juboori, R. A. (2014). Alternative methods of microorganism disruption for agricultural applications. *Applied Energy, 114*, 909-923.

Yusuf, R. O., Noor, Z. Z., Hassan, M. A. A., Agarry, S. E., & Solomon, B. O. (2013). A comparison of the efficacy of two strains of *Bacillus subtilis* and *Pseudomonas fragii* in the treatment of tannery wastewater. *Desalination and Water Treatment, 51*(16-18), 3189-3195.

Zbinden, M. D. A., Sturm, B. S., Nord, R. D., Carey, W. J., Moore, D., Shinogle, H., & Stagg-Williams, S. M. (2013). Pulsed electric field (PEF) as an intensification pretreatment for greener solvent lipid extraction from microalgae. *Biotechnology and bioengineering, 110*(6), 1605-1615.

Žgalin, M. K., Hodžić, D., Reberšek, M., & Kandušer, M. (2012). Combination of microsecond and nanosecond pulsed electric field treatments for inactivation of *Escherichia coli* in water samples. *The Journal of membrane biology, 245*(10), 643-650.

Zhan, Y., Martin, V. A., Geahlen, R. L., & Lu, C. (2010). One-step extraction of subcellular proteins from eukaryotic cells. *Lab on a chip, 10*(16), 2046-2048.

Zhang, C., Qi, J., & Cao, Y. (2014a). Synergistic effect of yeast-bacterial co-culture on bioremediation of oil-contaminated soil. *Bioremediation journal, 18*(2), 136-146.

Zhang, J., Fang, X., Zhu, X., Li, Y., Xu, H., Zhao, B., . . . Zhang, X. (2011). Microbial lipid production by the oleaginous yeast *Cryptococcus curvatus* O3 grown in fed-batch culture. *Biomass and Bioenergy, 35*(5), 1906-1911.

Zhang, K., Pei, Z., & Wang, D. (2016). Organic solvent pretreatment of lignocellulosic biomass for biofuels and biochemicals: a review. *Bioresource technology, 199*, 21-33.

Zhang, Q., Li, Y., & Xia, L. (2014b). An oleaginous endophyte *Bacillus subtilis* HB1310 isolated from thin-shelled walnut and its utilization of cotton stalk hydrolysate for lipid production. *Biotechnology for biofuels, 7*(1), 152.

Zhang, Y., Su, H., Zhong, Y., Zhang, C., Shen, Z., Sang, W., . . . Zhou, X. (2012). The effect of bacterial contamination on the heterotrophic cultivation of *Chlorella pyrenoidosa* in wastewater from the production of soybean products. *Water research, 46*(17), 5509-5516.

Zhang, Z., Jiaqiang, E., Deng, Y., Pham, M., Zuo, W., Peng, Q., & Yin, Z. (2018). Effects of fatty acid methyl esters proportion on combustion and emission characteristics of a biodiesel fueled marine diesel engine. *Energy conversion and management, 159*, 244-253.

Zhao, S., Wang, Y., Li, T., Zhang, S., & Cong, F. (2017). Exploration on Mixed Cultivation of *Lactobacillus*, Yeast and *Bacillus subtilis*. *Chemical and Biomolecular Engineering, 1*(2), 45.

Zhao, X., Hu, C., Wu, S., Shen, H., & Zhao, Z. K. (2011). Lipid production by *Rhodosporidium toruloides* Y4 using different substrate feeding strategies. *Journal of industrial microbiology & biotechnology, 38*(5), 627-632.

Zheng, Y., Yu, X., Zeng, J., & Chen, S. (2012). Feasibility of filamentous fungi for biofuel production using hydrolysate from dilute sulfuric acid pretreatment of wheat straw. *Biotechnology for biofuels, 5*(1), 50.

Zhu, L., Wang, Z., Shu, Q., Takala, J., Hiltunen, E., Feng, P., & Yuan, Z. (2013). Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment. *Water research, 47*(13), 4294-4302.

Zularisam, A., Ismail, A. F., Salim, M., Sakinah, M., & Hiroaki, O. (2007). Fabrication, fouling and foulant analyses of asymmetric polysulfone (PSF) ultrafiltration membrane fouled with natural organic matter (NOM) source waters. *Journal of Membrane Science, 299*(1), 97-113.

APPENDICES

APPENDIX A

FIGURES AND TABLES

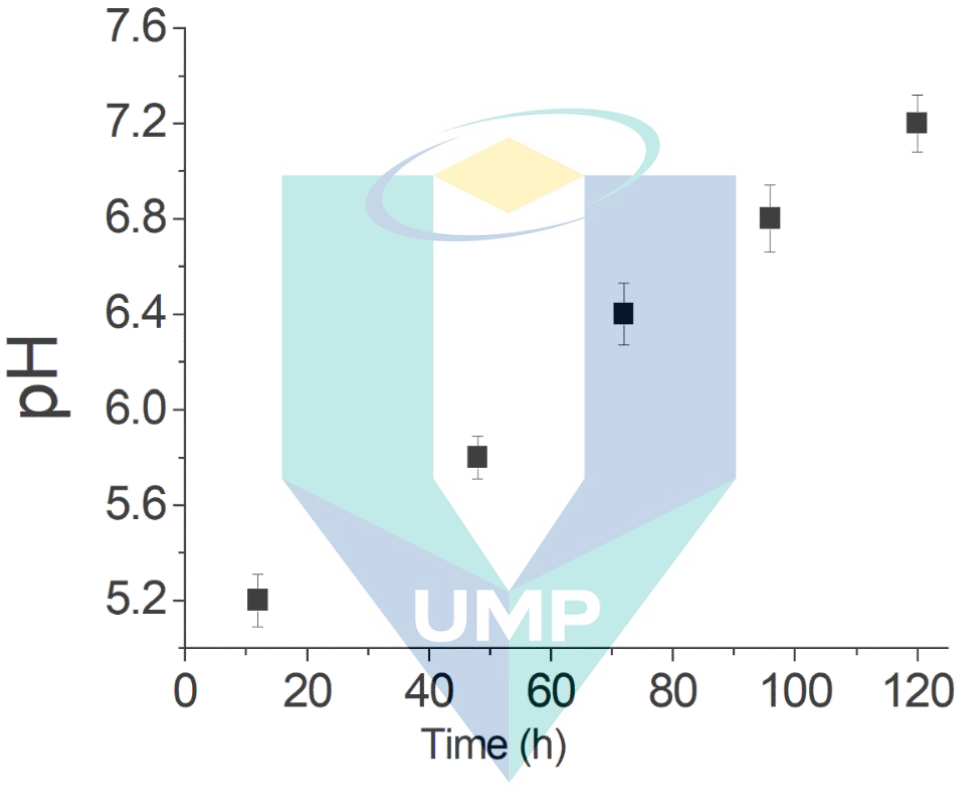

Figure A 1 Enhancement of pH during the treatment of POME.

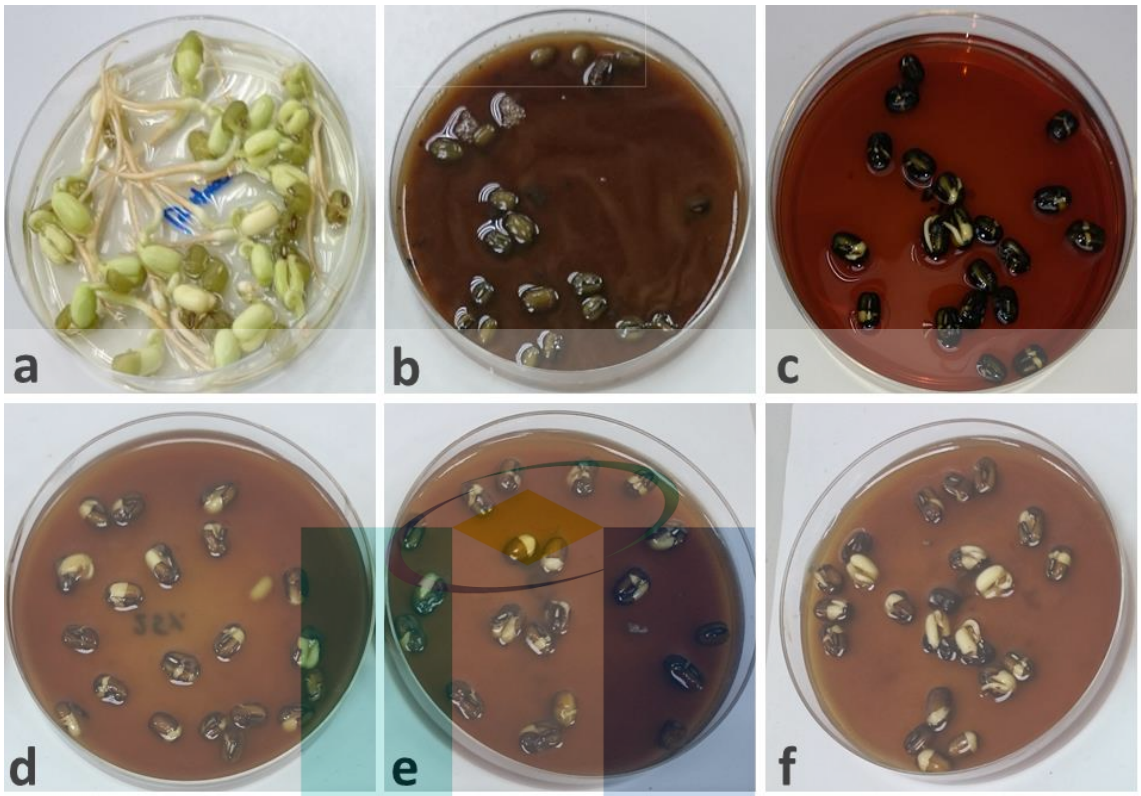

Figure A 2 Visualization of seed germination after 3 days on different POME medium a) control, b) raw (100% POME before treatment), c) 50% POME before treatment, d) 50% POME treated by *B. cereus*, e) 50% POME treated by *L. starkeyi*, f) 50% POME treated by co-culture.

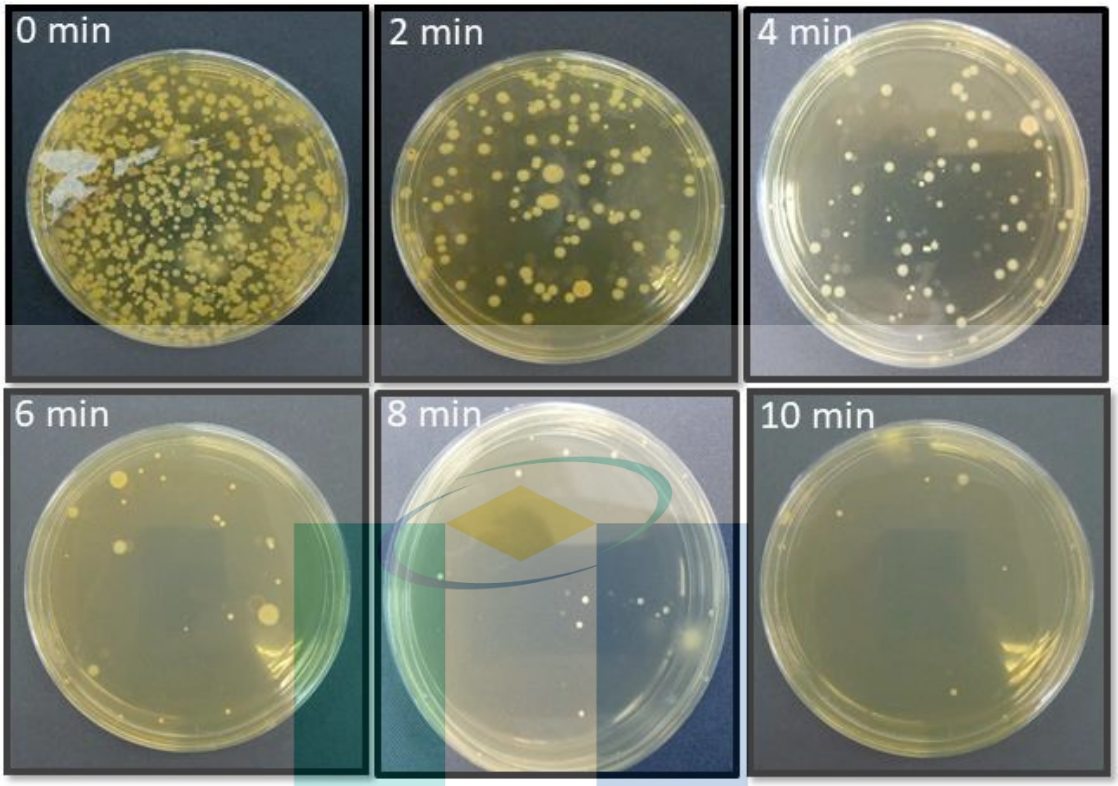

Figure A 3 Viability of colony forming unit (CFU) reduces with EP treatment (2 cm electrode distance).

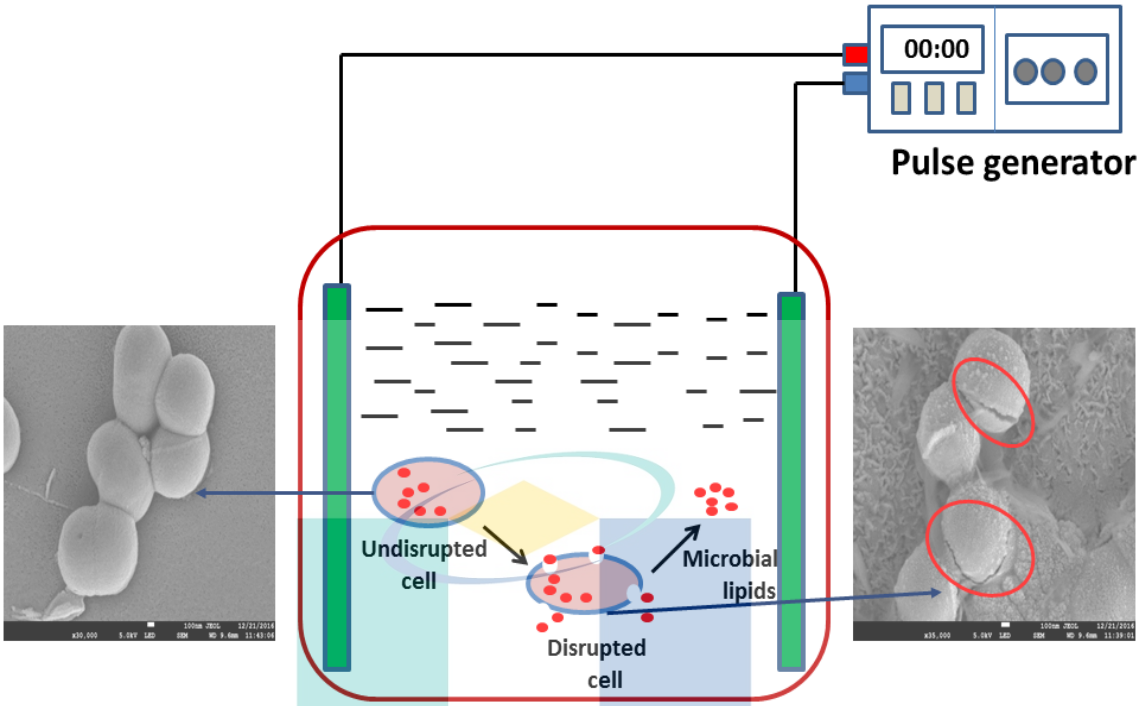

Figure A 4 Schematic of the EP treatment for lipid extraction from yeast cell.

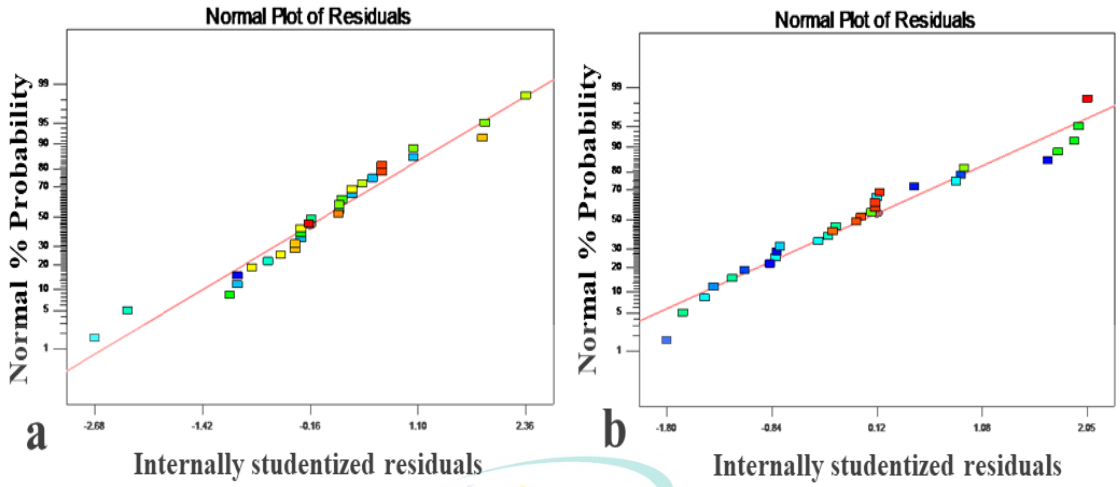

Figure A 5 Normal probability plot for the residuals from the (a) COD removal efficiency, (b) lipid accumulation output model.

Table A 1 Wastewater characteristics of raw POME and 50% POME (before and after autoclaved).

Parameter	Raw POME (mg/L)	50% POME	
		Before Autoclaved (mg/L)	After Autoclaved (mg/L)
Biochemical oxygen demand (BOD)	21600 ± 3150	16345 ± 1367	14821 ± 984
Chemical oxygen demand (COD)	52450 ± 5798	25752 ± 1648	23532 ± 762
Total organic content (TOC)	8900 ± 780	3873 ± 256	3485 ± 234
Total phenolic content (TPC)	2350 ± 670	1270 ± 195	1044 ± 87
Ammoniacal nitrogen (AN)	96 ± 22	67 ± 13	65 ± 9
Nitrite nitrogen (NN)	140 ± 32	84 ± 17	81 ± 12
Total nitrogen (TN)	752 ± 103	343 ± 57	328 ± 18
Total dissolved solid (TDS)	19875 ± 4795	11402 ± 2573	19687 ± 873
Total solid (TS)	35250 ± 12350	16035 ± 4187	35085 ± 996
Total suspended solid (TSS)	15360 ± 3275	6830 ± 1054	14963 ± 651
Oil and Grease (O & G)	4814 ± 2750	3857 ± 839	3523 ± 585

Table A 2 Central composite design (actual values) to maximize the COD removal and lipid accumulation using a co-culture inoculum.

Run No.	Concentration of inoculum A (%)	pH	Temperature (°C)	Incubation time (h)	COD removal (%)	Lipid accumulation (g/L)
1	50.00	6.50	32.50	90.00	85	2.81
2	30.00	7.00	30.00	80.00	73	2.05
3	50.00	6.50	37.50	90.00	80	1.97
4	50.00	5.50	32.50	90.00	74	1.76
5	90.00	6.50	32.50	90.00	75	1.13
6	30.00	7.00	30.00	100.00	78	2.18
7	50.00	6.50	32.50	90.00	83	2.77
8	30.00	6.00	30.00	100.00	81	2.25
9	70.00	7.00	35.00	100.00	82	1.58
10	70.00	7.00	35.00	80.00	75	1.14
11	30.00	6.00	30.00	80.00	73	1.67
12	30.00	6.00	35.00	80.00	76	1.99
13	30.00	7.00	35.00	80.00	80	1.14
14	50.00	7.50	32.50	90.00	83	1.57
15	50.00	6.50	32.50	90.00	84	2.80
16	70.00	6.00	35.00	80.00	80	1.58
17	70.00	6.00	35.00	100.00	82	1.78
18	30.00	7.00	35.00	100.00	82	1.48
19	70.00	6.00	30.00	80.00	73	1.25
20	70.00	6.00	30.00	100.00	78	1.55
21	30.00	6.00	35.00	100.00	81	1.76
22	50.00	6.50	32.50	90.00	82	2.71
23	50.00	6.50	32.50	90.00	83	2.76
24	10.00	6.50	32.50	90.00	73	1.25
25	50.00	6.50	32.50	70.00	75	1.56
26	50.00	6.50	32.50	110.00	86	2.89
27	70.00	7.00	30.00	100.00	79	1.25
28	50.00	6.50	27.50	90.00	70	1.37
29	50.00	6.50	32.50	90.00	85	2.80
30	70.00	7.00	30.00	80.00	73	1.16

APPENDIX B

LIST OF PUBLICATIONS

A. PULICATIONS

Journal articles:

Ahasanul Karim, M Amirul Islam, Abu Yousuf, Md. Maksudur Rahman Khan, and Che Ku Mohammad Faizal. (2019). Microbial Lipid Accumulation through Bioremediation of Palm Oil Mill Wastewater by *Bacillus cereus*. ACS Sustainable Chemistry & Engineering, ISI-index-Q1, **Impact factor: 6.97** (Published).

Ahasanul Karim, Abu Yousuf, M Amirul Islam, Yasir H Naif, Che Ku Mohammad Faizal, Md. Zahangir Alam and Domenico Pirozzi. (2018). Microbial lipid extraction from *Lipomyces starkeyi* using irreversible electroporation. **Biotechnology progress,** ISI-index-Q2, **Impact factor: 2.40** (Published).

Ahasanul Karim, M. Amirul Islam, Che Ku Mohammad Faizal, Abu Yousuf, Martin Howarth, Bipro Nath Dubey, Chin Kui Cheng and Md. Maksudur Rahman Khan. (2018) Enhanced Biohydrogen Production from Citrus Wastewater Using Anaerobic Sludge Pretreated by an Electroporation Technique. **Industrial & Engineering Chemistry Research,** ISI-index-Q1, **Impact factor: 3.37** (Published).

M. Amirul Islam, Abu Yousuf, **Ahasanul Karim**, Domenico Pirozzi, Md. Maksudur Rahman Khan and Zularisam Ab Wahid. (2018). Bioremediation of Palm oil mill effluent and lipid production by *Lipomyces starkeyi:* A Combined approach. **Journal of Cleaner Production**, ISI-index-Q1, **Impact factor: 6.39** (Published).

Conferences papers:

Ahasanul Karim, M Amirul Islam, Abu Yousuf, Md. Maksudur Rahman Khan and Che Ku Mohammad Faizal. Microbial lipid extraction by electroporation technique culturing *Lipomyces starkeyi* on palm oil mill effluent (POME). "International Journal of Advances in Science, Engineering and Technology (IJASEAT)". International Conference on Environment and Natural Science (ICENS)-2017, Dhaka, Bangladesh.

Book chapters:

Ahasanul Karim, Amirul Islam, Zaied Bin Khaled, Che Ku Mohammad Faizal, Md. Maksudur Rahman Khan and Abu Yousuf. Microalgal cell disruption and lipid extraction techniques for potential biofuel production. (2019). In Book: Microalgae Cultivation for Biofuels Production; 9:129-147, Publisher: Academic Press (Elsevier).

Ahasanul Karim, Amirul Islam, Shefa Ul Karim, Che Ku Mohammad Faizal, Maksudur Rahman Khan and Abu Yousuf. (2020). Dry fermenters for biogas production. In Book: Bioreactors for Bioenergy and Waste abatement; Publisher: Academic Press (Elsevier).

B. RESEARCH AWARD

1. **Gold Medal**, Creation, Innovation, Technology & Research Exposition (Citrex)-2017, Universiti Malaysia Pahang, Pahang, Malaysia. Project-"Irreversible Electroporation in Biofuel Production".